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THE HEMOPOIETIC RESPONSE IN THE CATFISH, AMEIURUS NEBULOSUS, TO CHRONIC LEAD POISONING

ALDEN B. DAWSON

(From the Biological Laboratories, Harvard University)

In studies of the action of soluble salts of lead on fresh water fishes, Carpenter (1927, 1930) found that concentrations of lead nitrate as low as Pb 1: 3,000,000 proved lethal. The speed of lethal reaction was dependent upon the total quantity of metallic ions present as well as upon the actual concentrations used, and varied in inverse ratio to the size and weight of the fish employed. The most marked symptom was the formation of a film over the gills and skin of the fish by the interaction of the metallic ions with the surface mucus, causing death by suffocation. When insufficient lead was present the film was shed and complete recovery took place. Chemical analyses showed that no metallic ions had penetrated the body itself and Carpenter held that the action was purely an external process, chemical in type, but mechanical in its effect. In these studies the exposure to lead was of relatively short duration and probably little of the metal had entered the body, although the test (H_2S) used to detect the presence of lead in the ash residue of the body contents (Aub, Fairhall, Minot and Reznikoff, 1926) is not extremely delicate.

It seemed of interest accordingly to determine the relative efficiency of the mucus secreted by the exposed surfaces of fishes in binding the lead as an inert compound. In the light of other observations made on the effects of lead-poisoning in *Necturus* (Dawson, 1933*b*) it was felt that a biological test, the injurious effect on the circulating erythrocytes, might prove more satisfactory than the chemical detection of the metal in the tissues and body fluids of the animal.

Material and Methods

Young catfish 4 to 5 inches in length were used in this study. The animals were kept continuously in a bath composed of 20 cc. of 1 per

cent lead acetate and 4 liters of tap water, which was changed every 48 hours. The experiments were begun in January and the experimental periods extended from 16 to 183 days. Samples of blood were drawn at regular intervals from a vein on the inner side of the operculum. The blood was studied in fresh preparation both by the Janus green-neutral red and the brilliant cresyl blue supravital techniques, and in dry smears stained by Wright's method. At the termination of the varying exposures to lead the animals and suitable controls were killed. The heart, liver, spleen, and mesonephros were removed, fixed in Helly-Zenker and stained with either hematoxylin and eosin or azur-eosin.

Effects of Lead Poisoning

Changes in the Peripheral Blood

In the catfish, practically all of the erythrocytes in the peripheral circulation are fully differentiated and basophilic or polychromatic cells are rare. In this respect they resemble those marine teleosts which are capable of removing dissolved oxygen from the water at a low oxygen tension (Dawson, 1933*a*). Exposure to lead produces no immediate or striking reaction within the blood stream. Intravascular phagocytosis of injured cells, such as occurred in *Necturus* (Dawson, 1930*a*), is almost entirely absent; the maximum number of active phagocytes ever observed in a study of any one supravital film (made with a circular cover-slip ¾ inch in diameter) being from 6 to 8. Injured cells appear smaller and become highly refractile; they are deeper in color, usually orange-yellow. Furthermore they are readily distorted, less elastic and consequently more fragile. In supravital preparations when slightly overstained with brilliant cresyl blue they appear green. With neutral red they are colored a deep red. Intracellular crystallization of hemoglobin, as in *Necturus* (Dawson, 1930*b*), is also readily induced by the slow drying of thick smears. Normally this does not occur although liberated hemoglobin crystallizes readily in this animal.

Following these early evidences of direct injury to the mature erythrocytes there is an increase in the number of polychromatic and basophilic cells, representing an exodus of incompletely differentiated erythrocytes from the erythropoietic loci, spleen, and mesonephros. At the end of thirty days a secondary anemia can be recognized and this increases in severity with the prolongation of the exposure to lead. Concomitant with the increased anemia, erythroblasts in varying stages of differentiation appear in the circulation and there is a marked increase in the number of small and large lymphocytes.

The cellular changes in the constitution of the blood, however, are not confined entirely to the cells of the erythrocytic series. Monocytes

and the large granular eosinophiles are increased in number, but the most conspicuous change occurs in the numbers and relative proportions of thrombocytes and spindle cells (Fig. 9). These elements seem to be identical with the two types of cell distinguished by Jordan and Speidel (1930) in the blood of cyclostomes. The thrombocytes have a specific, fine reddish granulation with a homogeneous outer cytoplasm. The spindle cells are long and fusiform, frequently with their cytoplasmic processes recurved. They may possess granules similar to those of the typical thrombocytes. Both cells usually possess a similarly grooved nucleus. Jordan and Speidel, after reviewing the evidence on the identity of these elements, were unable to decide whether they were genetically related or independent. The typical spindle form in the catfish is not assumed in fresh preparations and the two types of cell are not readily distinguished previous to drying on the slide. It seems possible that the spindle-form is an atypical thrombocyte.

In normal blood the number of spindle cells is very small but after several weeks exposure to lead the number is greatly increased and practically equals that of the thrombocytes. This condition, when established, persisted throughout the experiments. Young thrombocytes are frequently present in considerable numbers. Some are relatively large and many give evidence of amitotic proliferation. The appearance of these atypical cells, probably all of the thrombocytic series, may be correlated with the activity of the endothelium of the heart, which will be described later. The evidence is not conclusive.

The occurrence of injured erythrocytes, the progressive anemia and the marked regenerative response in the peripheral blood indicated that large numbers of erythrocytes were being removed from the vascular channels although only a limited amount of intravascular phagocytosis could be detected. However, additional evidence of the degree of erythrocyte destruction was obtained from an examination of the liver, spleen, and mesonephros. In these organs there was a progressive storage of the hemoglobiniferous residue of degenerated red cells. In the heart no such accumulations were observed, but a marked proliferative response of the endothelium was obtained.

Changes in the Organs

In a recent study Mackmull and Michels (1932) reported on the sites and mode of storage of colloidal carbon injected into the peritoneal cavity of a teleost, the cunner. One hour after the injection, the vascular channels transported free carbon and carbon macrophages to such organs as the heart, gill, spleen, intestine, testes, ovary, and kid-

ney. Extravascular migration of macrophages was most pronounced in the liver, spleen, kidney, and gonads.

The process of disposal of degenerate erythrocytes in the catfish parallels closely that of carbon storage in the cunner, especially in the liver, spleen, and mesonephros. Furthermore in the cunner, these organs normally contained collections of macrophages, the endogenous brown pigment of which was derived from degenerate red blood cells, and the migrating carbon macrophages displayed a selective orientation toward these areas of normal pigment deposit. However, in catfish, of the size used in this study, there are no such accumulations of stored pigment in these organs although a few isolated pigmented macrophages are occasionally found in their interstitial tissues. Accordingly the almost complete absence from the liver, spleen, and mesonephros of this pigment derived from dead erythrocytes furnishes a convenient base-line from which the amount of erythrocyte destruction may be determined by observing the progressive accumulation of pigment in the interstitial tissues by the macrophages.

Liver.—The liver of the catfish resembles that of the cunner (Mackmull and Michels, 1932), flounder, tautog, scup, minnow, and buffalo-fish (Jordan and Speidel, 1924) in that many of the veins are surrounded by sheaths of pancreatic tissue (Figs. 1 and 2). The pancreatic tissue is usually separated from the liver cells by a capillary space lined with reticulo-endothelium and frequently small foci of hemopoiesis may occur in the loose connective tissue in and about the intrahepatic pancreas. The progressive accumulation of pigment of erythrocytic origin within the liver is very marked (Figs. 3 and 4).

EXPLANATION OF PLATES

All figures are photomicrographs. Figures 1 and 3 were photographed under low power; Figs. 2, 4, 6, 7, and 8 under medium power; Figs. 5, 9, 10, 11, and 12 under oil immersion.

PLATE I

1. A low power view of an area of the liver from a normal fish, showing the general appearance of the hepatic cells as well as of the intrahepatic pancreatic tissue which borders the veins of the liver.

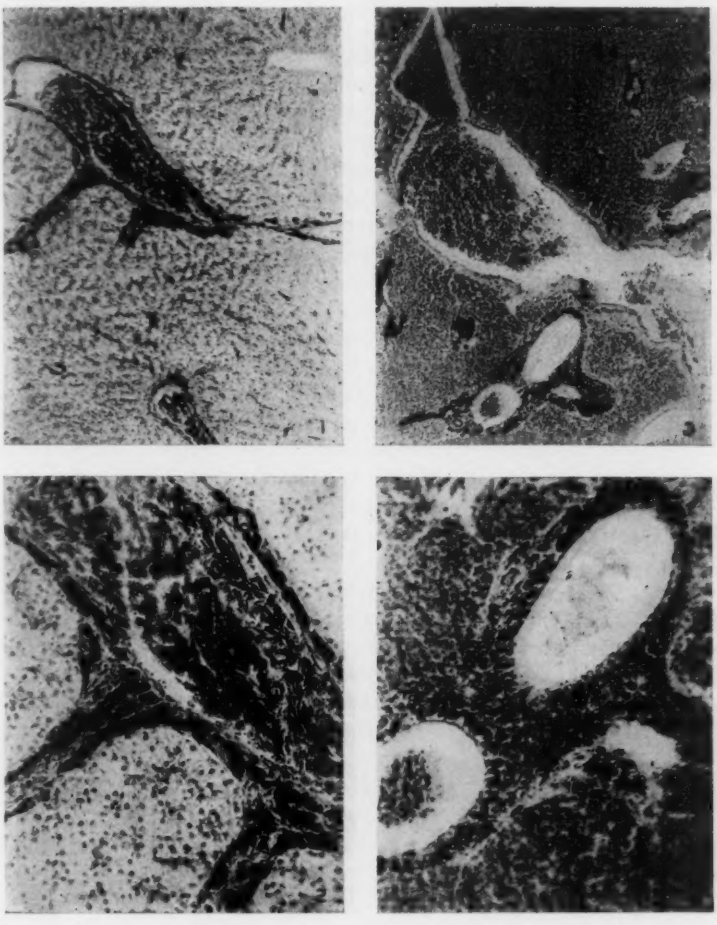
2. A portion of the above under greater magnification, showing the hepatic and pancreatic tissue in greater detail. Note the almost complete absence of brown and yellow granules (derived from disintegrated erythrocytes) in either the hepatic or interstitial cells.

3. A low power view of an area of the liver from a fish exposed to lead acetate for 183 days. The liver cells are small and dense and crowded with refractile yellow and brown granules. Masses of cells (macrophages) filled with similar pigment are scattered throughout the intertubular tissue. These cells are especially concentrated in and about the pancreatic epithelium.

4. A portion of the above under greater magnification, showing the concentration of pigment in the region of the pancreatic tissue.

The chief loci of storage are around the pancreatic cells although relatively large isolated groups of macrophages are also scattered throughout the organ between the hepatic cords. In addition to these sites of

PLATE I



pigment storage, every liver cell is crowded with numerous brown refractive granules which appear similar to the granular contents of the macrophages (Fig. 5), but there is an almost complete absence of

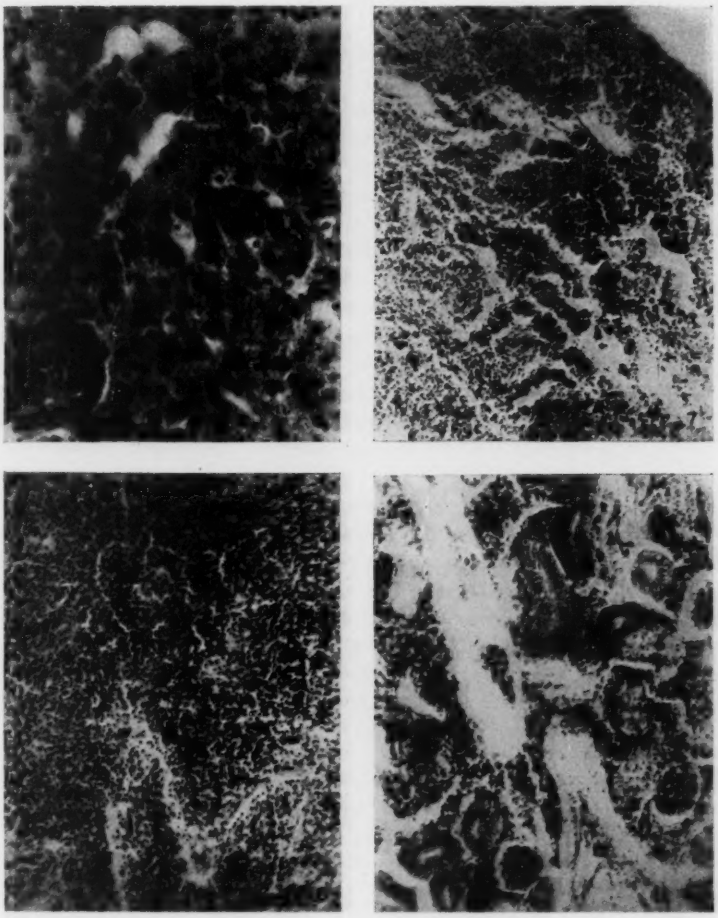
granular debris in the stellate cells of Kupffer, and in the endothelium of the veins associated with the pancreatic tissue. Only the lining of the capillaries in and about the pancreatic cells shows storage. Similar restriction of phagocytic activity of the vascular channels of the liver of the cunner was noted by Mackmull and Michels (1932) in carbon absorption. In fishes killed in late stages of lead poisoning the gall bladder is greatly dilated.

Spleen.—The general histological features of the spleen of fishes has been described by Yoffey (1929) and Mackmull and Michels (1932). The latter authors found that in the cunner the carbon macrophages and pigment macrophages migrated toward the splenic arterioles and occupied almost exclusively a periarterial position. As already pointed out in the normal young catfish, pigment macrophages are very rare, only a few isolated cells being present (Fig. 6). During lead poisoning the number progressively increases until the entire spleen is mottled with conspicuous yellow-brown masses (Fig. 7). Their distribution does not exactly coincide with that described for the cunner. Besides the definite concentration about the arterioles, there are equally large masses of pigment adjacent to the large splenic venules with which the arterioles are frequently closely associated. During lead poisoning the erythropoietic activity of the spleen is greatly reduced and few differentiating erythrocytes can be recognized in the pulp. The organ becomes smaller and paler.

Mesonephros.—The mesonephros in the teleosts is an important hemopoietic locus in which both erythrocytes and granulocytes are produced. The greater amount of hemopoietic activity is extravascular, occurring in the uniformly distributed loose intertubular connective tissue, but many of the peritubular capillaries show sinusoidal enlargements in which differentiating blood cells are also found. Pigment macrophages show no special groupings but local accumulations of such cells are scattered rather uniformly throughout the intertubular hemopoietic tissue. The progressive storage of this pigment during the exposure to lead is as evident in the mesonephros as in the liver and spleen but is never so extensive. No pigment was ever observed in the epithelium of the uriniferous tubules.

Heart.—Although pigment macrophages are commonly observed in the blood vessels of the spleen, liver, and mesonephros, they are not encountered either in the peripheral circulation or in the heart. They apparently move rapidly into the extravascular tissues of the organs and are not swept out into the general circulation. This is due largely to the fact that the degenerated erythrocytes are mostly phagocytosed by the attached reticulo-endothelial cells which later desquamate and enter the interstitial tissue.

PLATE II



5. A small area of hepatic tissue, showing the compact liver cells crowded with refractile granules. An interstitial mass of macrophages is seen in the lower, left corner.

6. An area from a normal spleen, showing the absence of pigmented macrophages.

7. An area from the spleen of an animal treated with lead acetate for 183 days. Note the massive infiltration of the splenic tissue by pigmented macrophages.

8. An area from the mesonephros from the same animal, showing the intertubular, extravascular concentration of pigmented macrophages. The mesonephric tubules are free of pigment.

In many of the lower vertebrates the endothelium and even the intermuscular connective tissue may display hemopoietic potencies. In the catfish the definitive lumen of the ventricle is small and the myocardium is made up of small muscle bundles separated by vascular channels, of the order and size of capillaries, which anastomose with one another to open eventually into the main ventricular cavity. The endothelium of these ramifying vascular channels is in intimate contact with the muscle fibers and in many instances does not appear to be sharply delimited from the intermuscular connective tissue.

In the cunner (Mackmull and Michels, 1932) both the endothelium of the minute intertrabecular vessels and the intermuscular connective tissue cells phagocytosed carbon. However, phagocytosis of injured erythrocytes was never observed in the heart of the catfish at any stage of lead poisoning and, as already noted, pigmented macrophages are also typically absent from the blood channels of this organ.

In the normal catfish the hemopoietic activity of the heart appears much less than in the cunner although small numbers of differentiating cells may occur in the capillary-like spaces of the myocardium. No lymphoid sheaths were observed. After several weeks of lead poisoning a marked proliferative response was obtained and relatively large clusters of cells were found on the surfaces of the muscle bundles (Fig. 10). The cells of such clusters are elongated and radiate from the site of proliferation into the lumina of the vascular channels. Their identity has not been definitely established, but it seems likely that they give rise to the atypical elongated and spindle cells described with the thrombocytes of the blood stream (Fig. 9). Presumably these clusters of cells arise by proliferation of the endothelium and there are some indications that they are secondarily invaded by cells of the intermuscular connective tissue.

PLATE III

9. Two areas from a blood smear of a fish exposed to lead acetate for 72 days, showing atypical elongated and spindle cells.

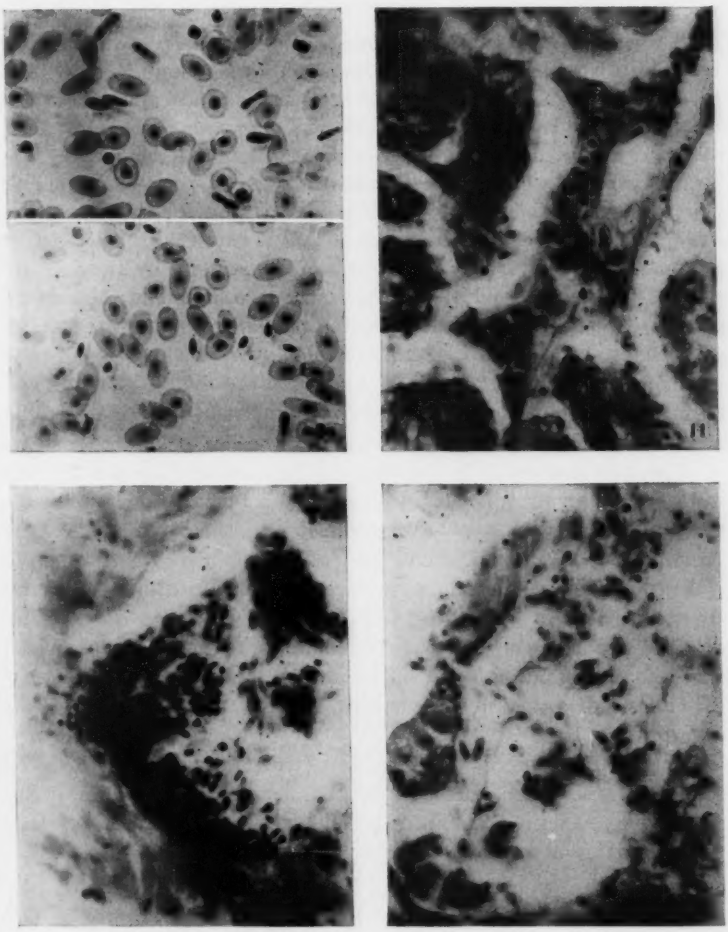
10. A region of proliferation from the surface of a muscular trabecula of the ventricle. The arrangement of cells resembles that in the margin of outgrowth of an explanted tissue. The elongated cells may give rise to the atypical cells provisionally classed with the thrombocytic series. The proliferating mass is apparently invaded by cells from connective tissue of the cardiac trabecula to form a reticular meshwork.

11. Another phase of the process of endothelial proliferation. The reticular meshwork is more obvious in this preparation. Small round cells and various stages of erythrocytic differentiation are present. Shrinkage has caused the separation of the tissue from the surface of the myocardial trabeculae.

12. This has been interpreted as a late stage of hemopoietic activity. The reticular meshes are distinct and almost empty but a few small round cells and erythrocytes are present. A row of small round cells remains attached to the surface of the trabecula.

In other regions of the heart less conspicuous areas of proliferation are also present. They apparently have the same relationship with the endothelium and connective tissues as the other proliferating cells,

PLATE III



but in the latter case various stages of erythrocyte differentiation can be readily recognized (Figs. 11 and 12). The reticular-like cells are also more readily observed in these regions. The connective tissue and

the endothelium of the ventricular wall of the catfish appear relatively undifferentiated, being essentially mesenchymal in nature and capable of hemopoietic activity although in this study they display no phagocytic activity toward the degenerate erythrocytes.

Discussion

These experiments with lead acetate furnish abundant evidence that teleosts such as the catfish slowly absorb lead when kept in solutions of this metal for relatively long periods. The surface mucus, while it may be effective in binding the lead for short periods (Carpenter, 1927, 1930), does not adequately protect the fish during long exposures. During exposures lasting for days and even weeks, it seems probable that lead in solution would eventually be also brought into contact with the epithelium of the digestive tract as well as with that of the gills and integument, and absorption may have been favored by this circumstance. In order to reduce this possibility to a minimum the animals were always removed to fresh tap water for feeding. However, there is no reason to believe that the mucin of the digestive tract would be less efficacious in binding the metallic ions than that of the gills and epidermis, but elimination of the mucous film could not be so readily accomplished in the digestive tract.

The observations on the peripheral blood demonstrate direct injury to the erythrocytes, followed by a mild regenerative response with the eventual development of a pronounced secondary anemia. The progressive storage of pigment, derived from phagocytosed erythrocytes, by the liver, spleen, and mesonephros also furnishes corroborative evidence of a widespread destruction of red blood cells. Since direct injury to the erythrocytes is generally the first and most important sign of lead poisoning in vertebrates, it seems safe to assume that the changes described in these experiments are the direct result of the absorption of lead.

The catfish apparently is less sensitive to lead than the fishes studied by Carpenter. Lead acetate rather than lead nitrate was used exclusively in my experiments, but no lethal effects were obtained although the concentration of lead was higher than that which proved lethal in Carpenter's series.

The changes in the peripheral blood of the catfish were comparable in many respects to those observed in an earlier study of lead poisoning in *Necturus*. The chief differences were in the absence of any considerable degree of phagocytic activity in the peripheral circulation and in the appearance in the circulation of large numbers of atypical cells,

elongated and spindle forms, which may belong to the thrombocytic series.

The mode of accumulation and storage by the liver, spleen, and mesonephros of the pigment derived from the injured red blood cells follows closely that described in the elimination of particulate matter from the circulation of fishes. No storage occurred in the heart but a proliferative response to the destruction of the circulating elements was obtained. The erythropoietic potentialities of the cardiac endothelium of the lower vertebrates is well known and the literature is well summarized by Mackmull and Michels (1932). Indeed, in such forms as the herring (John, 1932) it may be the initial site of erythropoiesis. There are no red blood cells in the larval stages and they appear several months after hatching.

No evidence of direct injury to the thrombocytes was obtained and it is difficult to explain either the increase in number of the cells of this series or the atypical response of the cardiac endothelium in their production. The production of thrombocytes and erythrocytes usually takes place in the same organs or tissues and the two processes go on side by side. Furthermore the two types of cell are quite similar in their plan of organization. It appears possible that any factor which depresses or modifies erythropoiesis may tend to exert a similar influence on thrombopoiesis.

Summary

In the catfish, with prolonged exposure to a solution of lead acetate, definite evidence of absorption of lead was obtained. The surface mucus did not constitute an efficient barrier to the entrance of the metal as Carpenter (1927, 1930) found in more acute experiments with fishes in which a lethal reaction was frequently obtained.

The degree of destruction of the erythrocytes was used as a measure of the rate of the absorption of lead. Following the early evidences of injury to the blood cells, a mild regenerative response occurred, but eventually a pronounced secondary anemia was produced. Little phagocytosis of dead cells occurred in the peripheral circulation, but a progressive storage of the pigment derived from dead red blood cells was found in the interstitial tissues of the liver, spleen, and mesonephros. The hepatic cells also became crowded with pigment granules. No storage was observed in the heart. The pigment was accumulated chiefly in macrophages of local origin which, after ingesting erythrocytes, desquamated and migrated into the connective tissues.

Monocytes and eosinophiles were increased slightly in number in the blood stream, but the most striking change occurred in the numbers

of atypical thrombocytes, and spindle cells which may belong to the thrombocytic series.

The endothelium of the heart showed marked proliferative activity. In some regions there was localized differentiation of erythrocytes. In other regions the atypical elongated cells, just referred to, were formed in large radiating clusters on the surface of the ventricular trabeculae. No explanation of the latter response to lead poisoning can be given.

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THE RELATION BETWEEN CELL INTEGRITY AND BACTERIAL LUMINESCENCE

IRVIN M. KORR

(From the Physiological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole, Mass.)

In 1902 Macfadyen found that luminous bacteria exposed to liquid air would glow again on rewarming, but gave no light on rewarming if thoroughly ground at the temperature of liquid air. Harvey (1915) tried to obtain luminescence from cytolyzed and dried crushed bacteria, but even though the possibility of oxidation of the bacterial luciferin by molecular oxygen was precluded (a precaution Macfadyen did not take) by grinding the bacteria in the desiccated form or by cytolyzing them with toluol, ether, water, and other agents in the absence of oxygen, no light was obtained on moistening the ground bacteria, or aerating the cytolysate. Intact, dead, desiccated bacteria luminesced for a short time when moistened, even after prolonged extraction with fat solvents such as ether, absolute alcohol, toluol, benzine, and chloroform. These agents acting on moist or suspended bacteria, however, invariably and quickly destroyed permanently the ability to luminesce.

The demonstration of a given reaction in cellular extracts has always been a much more difficult problem in the case of bacteria than in other cells, due to the relatively greater importance of the cell structure. According to Quastel (1926) catalytic dehydrogenations, particularly, are very closely associated with the surface of the bacterial cell.

The demonstration of luminescence apart from cell structure is an even more difficult problem since it is necessary to extract both the catalyst, luciferase, and its specific substrate, luciferin. The normally close association between the two is very likely disturbed, due, conceivably, to differences in solubility between the two, to the instability of one or both apart from the normal structure of the cell, or to the oxidation of the luciferin by oxidants other than oxygen.

New methods have been applied in a continued investigation of this problem, namely, the attempt to demonstrate luminous substances from luminous bacteria destroyed in various ways. Three species were used: a fresh-water form, *Vibrio phosphorescens*, and two marine

forms from Woods Hole, one unidentified, and the other, *Achromobacter fischeri*, a very brilliant variety. Four general methods, with numerous modifications, were employed for the destruction of the bacteria: (*a*) cytolysis by fat solvents, (*b*) cytolysis with hypotonic solutions (not applicable, of course, to the fresh-water form), (*c*) mechanical grinding, and (*d*) sonic radiation.

Throughout these experiments care was taken to prevent oxidation of the luciferin, and, hence, they were carried out anaerobically. After destruction of the bacteria, the suspensions were aerated in the dark and observations on the presence or absence of luminescence made with completely dark-adapted eyes.

Experiments with Fat Solvents

For this series of experiments and those involving osmotic cytolysis a double trap vessel illustrated in Fig. 1 was found convenient for the anoxic mixing of cytolytic agents with the suspension. The agent was put in one arm of the vessel (*B*), the suspension in the other (*A*), and thoroughly deaerated with a stream of hydrogen purified by passage over red-hot platinized asbestos. The two were then mixed by tilting the vessel. On aerating, no return of luminescence was observed with either chloroform or caprylic alcohol as the cytolytic agent.

A modification of this technique, in which deaeration was accomplished with sodium hydrosulfite, yielded the same results.

These confirm Harvey's (1915) results: suspensions deaerated by evacuating and then cytolyzed with toluol, ether, chloroform or carbon tetrachloride showed no luminescence on re-aerating.

Experiments with the Osmolytic Technique

The cytolytic agent in this series is diluted sea water. A volume of distilled or tap water found, in air, to cause rapid darkening of a given volume of a dense sea-water bacterial suspension is pipetted into one arm of the vessel (*A*), and the suspension into the other (*B*). The rest of the experiment is run as in the above series. Although repeated many times, luminescence has never been obtained from bacteria "cytolyzed" by hypotonic solutions in the absence of oxygen.

Darkening of bacterial suspensions by hypotonic solutions is perhaps not a true indication of complete cytolysis of the bacteria. Although the suspensions do become clear and foamy, due to the release of cellular contents almost immediately after mixing with distilled water, the bacteria remain viable for hours after complete darkening, as shown by inoculating culture media with portions of the suspension

at intervals after complete darkening.[1] Excellent growth will occur after 3½ or more hours exposure to distilled water. Even though true cytolysis by this method is a very slow process, the copious release of cellular contents justifies its application.

It is possible that, despite the most rigorous exclusion of oxygen, the bacterial luciferin, during release from the cell, becomes oxidized (by the more positive systems in the cell) without giving light. Harvey

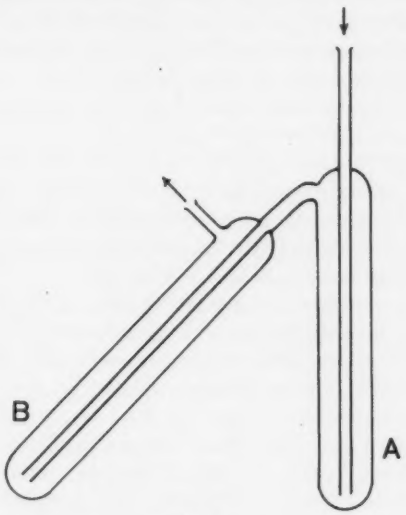

FIG. 1.

(1927) has shown that *Cypridina* luciferin can undergo non-luminescent (and reversible) oxidations not involving molecular oxygen. Strong reducing agents should prevent such oxidations, but the presence of finely divided Pt in the cytolyzing mixture, which, together with the hydrogen used for deaerating, gives a reducing intensity approaching that at a hydrogen electrode, does not alter the results. The same is true if the osmotic cytolysis is allowed to take place in the presence of a

[1] The darkening of bacterial suspensions by hypotonic solutions is doubtless at least partly an osmotic phenomenon, but not entirely, for a volume of tap or distilled water which causes rapid darkening of a given volume of suspension will do so only if added at once. If slowly poured in or added in three or four installments, even closely spaced, the time for darkening is greatly extended, the suspension retaining some luminescence for hours. Another recent and pertinent observation is that the destruction of the capacity for luminescence by hypotonic solutions is very much more rapid under anaerobic conditions than in aerobic. The difference is far too large to be ascribed merely to a difference in the rates of penetration of the water in the presence and absence of oxygen.

small but adequate mount of neutralized sodium hydrosulfite. *Cypridina* oxyluciferin, which gives no light with luciferase, can be reduced by hydrosulfite to luciferin, which does give light with luciferase.

Another possible cause of failure to obtain luminescence from substances liberated from bacteria is that the photogenic substances become diluted or separated, even when liberated into small volumes of liquid, whereas, in the cell, they are quite concentrated and closely associated. The attempt was made in the following experiments to minimize this dispersion by concentrating the substances on adsorbing surfaces. The procedure was exactly that described above for the osmolytic technique, except that an adsorbent, either $Fe(OH)_3$, kaolin, or Norit A was added to the distilled water. For the use of $Fe(OH)_3$ the bacteria had to be suspended in isotonic sucrose to prevent the too rapid precipitation of the sol by the salts of sea water on mixing. In no case was luminescence observed on aeration.

Repetition of these experiments but with the addition of finely divided Pt to both tubes to provide reducing action (together with the H_2) gave the same results.

In some experiments the adsorbents were replaced by fresh-water luminous bacteria from 4–6-day-old cultures which had ceased luminescing, or other fresh-water (non-luminous) forms. But the presence of normal bacterial surfaces instead of those of the colloids did not affect the results.

Attempts were also made to reduce oxyluciferin, liberated from cells by aerobic cytolysis. A thick mass of bacteria (*A. fischeri*), washed and collected by centrifuging, was suspended in water, in aqueous colloidal $Fe(OH)_3$ or in aqueous kaolin suspensions, in air. Some time after complete darkening of the suspension (due to hypotonicity) it was again centrifuged, the supernatant fluid poured off and the thick gelatinous mass at the bottom of the tube quickly dried in vacuo over $CaCl_2$. The dried powder was suspended in water and, in the absence of oxygen, exposed to the action of colloidal $Pt + H_2$. This (again assuming a fundamental similarity between bacterial and *Cypridina* luciferin) should have reduced the oxyluciferin formed during its (aerobic) release from the cell to luciferin. However, no light appeared on aeration in any case.

Autolytic Method

A dense mass of *A. fischeri* was kept at 38° C. for 3 days in order that autolysis might occur. This material, after reduction with $Pt + H_2$ or with hydrosulfite, gave no luminescence on aeration. The autolysate, which should have contained large quantities of bacterial

luciferase (and oxyluciferin), when mixed with bacterial luciferin, i.e. with what would be analogous to a *Cypridina* luciferin preparation (bacteria boiled and cooled in a hydrogen atmosphere), gave no light. Attempts to demonstrate luciferase in ground bacteria, desiccated marine bacteria resuspended in water or adsorption extracts, described in the preceding paragraph, with the above luciferin preparation also proved negative. Likewise, "cross" experiments with *Cypridina* luciferin and luciferase also failed.

Extracts of *Cypridina* which have become dark due to the oxidation of all the luciferin will, when electrolyzed, luminesce in the vicinity of the cathode, due to cathodal reduction (Harvey, 1923). Various extracts of the luminous bacteria, similarly treated, did not luminesce.

Mechanical Destruction of Bacteria

This was accomplished with a device consisting of a ground-glass rod revolving in a close-fitting glass tube whose inner surface in contact with the rod is also ground.[2] (Ten Broeck, 1931; Glaser and Coria, 1935.) The rod was fitted to the shaft of a small electric motor. In order to grind in the absence of oxygen, the grinder and motor were hermetically enclosed in a casement of brass and glass. The glass part consisted of a test tube sealed with deKhotinsky cement into a circular opening in the brass case containing the motor. This glass tube contained the grinding parts, thus permitting observation. Wires to the motor and gas-inlet and -outlet tubes were fitted to the brass case through a rubber stopper, so that the apparatus could be exhausted with a vacuum pump or hydrogen passed through it while grinding was taking place.

A small quantity of thick, bright suspensions was carefully pipetted into the bottom of the grinding vessel without getting bacteria on the walls of the vessel. The bacteria were ground either in an atmosphere of hydrogen or in a vacuum at a high rate of speed for one to two hours, after which air was admitted in the dark and observations made. The temperature never rose above 28° C. If grinding was thorough, no light appeared on aerating. If, before grinding was begun, finely divided Pt was added to the suspension, and the experiment performed in a H_2 atmosphere, the results were also the same, no light appearing on aeration.

Experiments with Sonic Vibration

A number of experiments in which destruction of the bacteria was accomplished by means of a powerful magnetostriction oscillator of

[2] The author is grateful to Dr. R. W. Glaser of Rockefeller Institute, Princeton, for a set of the glass parts, after which others were modelled.

9,000 cycles were performed.[3] Sonic radiation from the apparatus used is known to break apart bacteria into fragments (Chambers and Gaines, 1932) and will extinguish the luminescence of bacterial suspensions in air in 13 to 20 minutes. The suspension was contained in a glass vessel fitted over the vibrating nickel-alloy tube, and deaerated by a stream of nitrogen first passed over heated copper to remove traces of oxygen. The suspension was completely darkened by the removal of dissolved oxygen before the generator was turned on, but for some reason the suspension became slightly luminous again on even momentary irradiation, indicating the presence of a small amount of oxygen. Although this was washed out by the continued stream of N_2, there was almost always a reappearance of luminescence on irradiating. Bacteria, irradiated in the presence of this small concentration of oxygen for 13–20 minutes (the time required depending on the concentration of bacteria) showed no luminescence on blowing air through their suspensions. In only two experiments were we successful in completely deaerating the suspensions (as shown by darkness of the suspensions on momentarily turning on the oscillator). These, also, did not luminesce on being aerated at the end of the run.

The work of Shoup (1929, 1933, 1934) and of Taylor (1932, 1934) has shown that when respiration is inhibited by a variety of methods, e.g. KCN, CO, excessive dinitrophenol, lowered oxygen tension, luminescence is not affected until respiration is reduced to about 40–50 per cent of the normal, when the intensity drops off. Whether the failure of respiration and of luminescence is due to some common factor or whether the failure of respiration bears some causal relation to that of luminescence cannot be readily determined. It is easy to show that the respiration of "cytolyzed" bacteria is greatly affected. As determined by a modification of the dimming-time method their respiration is reduced to far below the 40–50 per cent level, often lower than 10 per cent. It seems that the dehydrogenases are the affected systems, rather than the oxidase-cytochrome system, for the rate of reduction of methylene blue (Thunberg technique) is greatly lowered by osmotic cytolysis while the Nadi test for indophenol oxidase is, if anything, intensified.

Discussion

Even a cursory review of the literature shows that bacterial luminescence is far from unique among biological oxidative reactions in being dependent upon cellular structures and having catalysts not

[3] I am most grateful to Dr. Leslie A. Chambers and the Johnson Foundation for Medical Physics for the use of the oscillator and assistance in its operation.

extractable in the soluble form. Some of the oxidative reactions of blood cells are inhibited or destroyed by osmotic hemolysis, and it is particularly true that the dehydrogenations by bacteria disappear with cell destruction (Quastel and Woolridge, 1927, 1928; Stephenson, 1928; Young, 1929), although some of the dehydrogenations are less dependent on normal structure than others. Other interesting observations are those of Gozony and Suranyi (1925) and of Shwartzman (1926), who found that lysis with phage greatly reduced the rate of methylene blue reduction by bacteria. Clifton (1933) found that lysis with phage causes less negative reduction potentials in suspensions of *Staphylococcus aureus*. I have found that cytolysis (by hypotonic solutions, cytolytic agents, and autolysis) produces similar effects on luminous bacteria. The oxidative reactions observed by Neill and his coworkers (1924–1928) in detritus-free extracts of *Pneumococcus* are all very likely due to the presence of non-enzymic catalysts such as certain intracellular reversibly oxidizable pigments.

Whether luciferase can be classed with the dehydrogenases is, of course, debatable. One fundamental similarity is shown, however, in that luciferin (LH_2) is oxidized to oxyluciferin (L) by a dehydrogenation catalyzed by luciferase. The energy derived from this oxidation is then transferred to the luciferase, which luminesces (Harvey, 1927). *Cypridina* luciferin, however, differs from other hydrogen-donators in being autoxidized in the presence of oxygen, and readily oxidized by certain oxidizing agents, e.g. ferricyanide and quinone, but without the production of light. Hence, for luminescence, the reaction is highly specific.

Although bacterial luminescence and *Cypridina* luminescence are both dependent on molecular oxygen, the inability to demonstrate the luciferin-luciferase reaction or to obtain any luminescence from injured or disintegrated bacterial cells, even under the most favorable conditions, forces us to the conclusion that luminescence in these forms is very decidedly bound up in an intimate manner with structural conditions, presumably unaltered surfaces in or on the cell.

Summary

Three species of luminous bacteria were injured or destroyed by a variety of modifications of four general methods: (*a*) cytolysis with fat solvents, (*b*) osmotic cytolysis, (*c*) mechanical grinding, and (*d*) intense sonic vibration. Although all experiments were performed under conditions which would have prevented the oxidation of the bacterial luciferin and which were, in general, favorable for bioluminescence, it was not possible in any case to demonstrate the luciferin-luciferase

reaction or obtain luminescence from bacteria whose structure had been materially altered. The conclusion is drawn that bioluminescence, like many other bacterial oxidative phenomena, is closely associated with cellular structure. Respiration and reducing activity were shown also to be greatly affected.

The author wishes to express his profound gratitude to Professor E. Newton Harvey, under whom this work was done, for his interest and helpful advice.

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THE EFFECTS OF ANTUITRIN S AND SHEEP PITUITARY EXTRACT ON THE FEMALE LIZARD, ANOLIS CAROLINENSIS

LLEWELLYN THOMAS EVANS

(From the Biological Laboratories, Harvard University)

Introduction

The relationship between the pituitary and the female genital system of the reptile is only partially understood. Ovulation in the snake, *Xenodon merremi*, resulted after five homoplastic pituitary implants (Houssay, 1931). Ovulation was induced in *Anolis carolinensis* after five homoplastic pituitaries were implanted and in one case after three frog pituitaries were implanted (Evans, 1935). Forbes (1934) reports hypertrophy of the genital system of young alligators with whole gland sheep pituitary extract.

Since homoplastic pituitary implants induce ovulation in reptiles, the present study was made in order to learn whether mammalian pituitary extracts would also induce ovulation.

The oviduct of the normal female of *Anolis carolinensis* is divided into five general regions: the infundibulum, the tube, the albumen-secreting portion, the uterus, and the vagina (Giacomini, 1893). The epithelial cells of the albumen-secreting portion are cuboidal and seem to be the only glands present, there being none beneath the mucous layer. Special glands, however, exist in the submucous region of the uterus. These connect with the lumen of the uterus by means of ducts.

Materials and Description

Antuitrin S Series

Fifty-five females of *Anolis carolinensis* received injections of Antuitrin S (Parke Davis, Series 3024898). The first injections were administered on November 22, 1933, the last on April 1, 1934. Some females received more injections than others and at varying intervals, but the dosage in all cases was .02 cc. diluted with two or three parts of cold-blooded Ringer's solution. This dose represents the maximum that was safe to use. Larger dosage often proved fatal. Twenty-five females served as controls and were kept under the same conditions as the injected animals.

Internal changes brought about by the injections were largely con-

fined to the ovaries and oviducts. Figure 1*a* shows the control (on the left) as compared to 1*b* from a female which received three daily injections, was then rested for 30 days, followed by six more injections, then another rest of 7 days followed by two injections. She was killed 24 hours later, February 28, 1934, together with the control shown in Fig. 1. Figure 1*c* shows the condition produced in a female which received twelve daily injections and then was killed, 24 hours later, March 15, 1934. These two particular cases are representative of the results obtained in the 55 females, so it seems unnecessary to illustrate other cases.

Reference to Fig. 1 reveals the alteration produced in the oviducts by injections. In the control the oviduct appears as a continuous straight tube which becomes slightly larger in the region of the uterus. Figures 1*b* and 1*c*, however, show the oviduct very much enlarged and thrown into many folds. The albumen-secreting portion becomes greatly lengthened but its walls remain relatively thin. The uterus, on the contrary, increases more in circumference than in length. This increase in diameter is brought about partly by a thickening of the walls due to the greatly hypertrophied glands lying beneath the mucous lining of the lumen. The infundibulum (*i*), albumen-secreting portion (*al*), and the uterus (*u*) are well shown.

The lumen of the infundibular region is usually closed by the temporary fusion of the mucous walls but, under the influence of the extract, the lumen becomes patent. The epithelial cells lining the tube region are not visibly hypertrophied by the hormone. The mucosa of the albumen-secreting portion shows considerable response. The individual cells change in shape from cuboidal to columnar.

EXPLANATION OF PLATE I

Abbreviations: *al*, albumen-secreting portion of oviduct; *g*, glandular area of uterus or shell-secreting portion of oviduct; *i*, infundibulum of oviduct; *l*, lumen; *m*, smooth muscle layer of oviduct; *s*, mucous layer of oviduct.

All figures are of *Anolis carolinensis*.

FIG. 1. 4¾ ×. Ovary and oviduct. (*a*) Control. Killed February 28, 1934. (*b*) Received injections of Antuitrin S with intervals of rest as follows: three daily injections, 30 days rest, 6 daily injections, 7 days rest, 2 daily injections. Killed February 28, 1934. (*c*) Received 12 daily injections of Antuitrin S. Killed 24 hours later, March 15, 1934. (*d*) Received 12 injections of whole gland sheep pituitary extract at 36-hour intervals. Killed 24 hours later, March 15, 1934.

FIG. 2. 210 ×. Control. Cross-section of albumen-secreting portion of the oviduct.

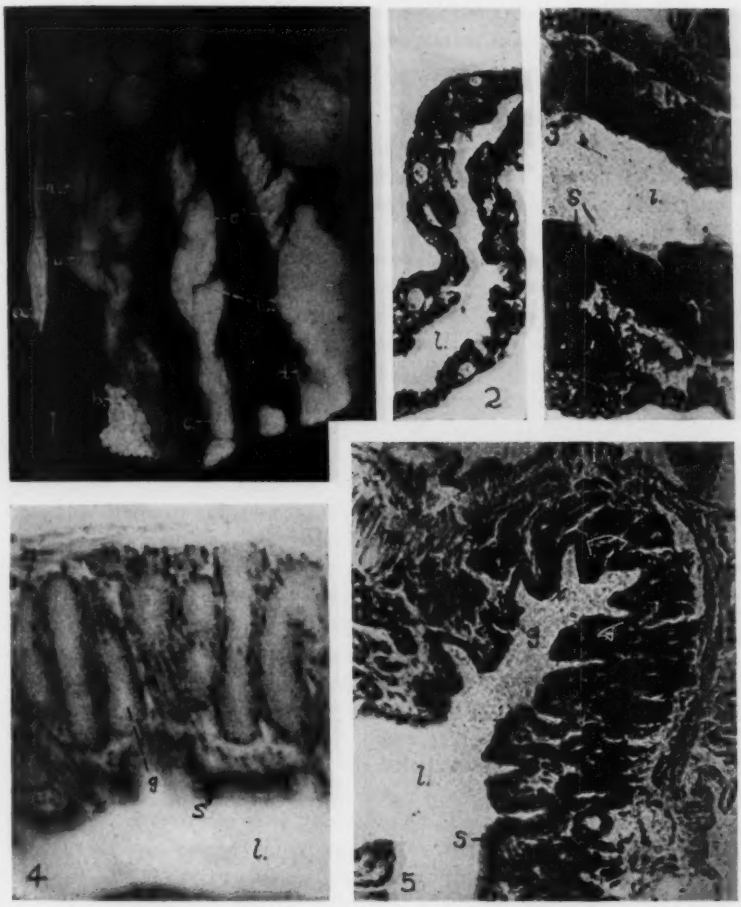
FIG. 3. 210 ×. Same as *d* in Fig. 1. Cross-section of albumen-secreting portion of the oviduct.

FIG. 4. 210 ×. Same as *d* in Fig. 1. Cross-section of uterus. Note duct from glandular area at *x*.

FIG. 5. 210 ×. Control. Cross-section of uterus. Note duct from glandular area at *x*.

The uterus shows a curious contrast to the albumen-secreting portion in its response to the injections. The superficial cells of the mucosa are not altered in shape but remain cuboidal. The so-called

PLATE I



shell-secreting glands which lie between the mucous layer and the muscle layer become much enlarged.

The ovary is definitely affected by Antuitrin S as Figs. 1*a*, 1*b*, and 1*c* bring out clearly. Figure 1*c* shows approximately 100 per cent hypertrophy as compared with the control. In Fig. 1*b*, however, the ovary is at least four times larger than the control.

It is interesting to note that eleven injections in groups of 3, 6, and 2, with several days interval between injections, produced a greater hypertrophy of both ovary and oviduct (1*b*) than was secured when one injection was given daily for twelve days (1*c*). Seasonal effects can be ruled out since 1*b* was killed two weeks earlier than 1*c*.

No matter what combination of injections and rest intervals was used, Antuitrin S failed to induce egg-laying.

Sheep Pituitary Series

Twenty-five females of *Anolis carolinensis* were injected with sheep pituitary (whole-gland pituitary extract [1]) at varying intervals between December 1, 1933 and April 1, 1934 and with a varying number of injections. A single dose was always .02 cc. diluted as for Antuitrin S. The same controls were used.

The use of sheep pituitary gave results which were more satisfactory in that complete ovulation was induced. On March 23, 1934 two eggs were laid and on March 24, two more. A fifth appeared on April 11. In these particular cases, twelve injections of sheep pituitary were given at an average of 36-hour intervals, the last injection being on March 2, 1934. All of the 25 females that received the sheep pituitary responded by a great hypertrophy of the ovaries and oviducts. Many cases showed, however, that the eggs which were ready to be laid were retained in the ovary. Such females had unusually large abdomens, moved about very little, and seemed to be in a lethargic state. In these cases the eggs were very slowly resorbed so that by the first of August the ovaries were reduced to the size of that shown in Fig. 1*c*. The bright orange color of the partially resorbed eggs made them easily distinguishable from young ova of the same size which were always white.

Figure 1*d* shows a typical case of induction. This particular animal was killed March 15, 1934, 24 hours after receiving twelve injections at 36-hour intervals. The large egg is almost ready to leave the ovary. The infundibulum has surrounded the egg, enveloping it like a transparent membrane. The latter is visible in the fresh condition only because of its blood vessels, which form a network over the egg (not visible in the photograph). The same figure shows clearly the infundibulum, albumen-secreting portion, and the uterus. Comparison with the normal oviduct at the time of ovulation (not shown) makes it seem certain that this figure represents a condition homologous to that just prior to normal ovulation.

Figure 1*d* represents, then, the maximum degree of hypertrophy which was obtained in our injected series. The uterine portion is

[1] Kindly supplied by Dr. Oliver Kamm of Parke Davis Company.

especially large. This hypertrophy is due primarily to the increase in the size of the glands which lie in the submucous region. These are larger in all dimensions than the same glands in Fig. 1*b* or 1*c*. Figure 4 shows a section of this uterus in greater detail. The glandular cells are palisade in shape and are filled with a highly refractive granular substance. The nuclei lie at the outer periphery of the cells and away from the lumen of the gland. The cells have become so enlarged that the free secreting surfaces of opposite cells abut one another, thus closing the lumina of the glands.

Non-sexual Effects of Both Antuitrin S and Sheep Whole Gland Extract

These effects made their appearance after the third or fourth injection and continued for at least four months after the last injection. Both extracts caused the females to differ from the controls in that (1) their appetites were greater and while they ate more they remained much thinner; (2) general activity and speed of movement were greater; (3) moulting occurred oftener.

Summary

Between November 1933 and April 1934, 55 females of *Anolis carolinensis* were injected with Antuitrin S, while 25 were injected with whole gland sheep pituitary extract. Twenty-five females served as controls. The dosage was .02 cc. of either extract diluted with cold-blooded Ringer's solution.

Both extracts caused hypertrophy of the ovaries and oviducts but ovulation and egg-laying were induced only with sheep pituitary. Moreover, in many females which received sheep extract the ovaries contained mature ova that were not laid but were slowly resorbed during the ensuing spring and summer.

With both extracts the epithelial cells lining the albumen-secreting portion of the oviduct changed from cuboidal to columnar, while those of the epithelium of the uterus were very little affected by the injections. The deep-lying shell glands of the uterus, however, were greatly enlarged.

Injected animals ate more, were more active, and moulted oftener than controls.

I wish to thank Professor Leigh Hoadley and Professor Alden B. Dawson for their kind help and valuable criticism during the course of this investigation.

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THE HEMOLYTIC ACTION OF PHOTOFLUORESCEIN [1]

JOHN F. MENKE

(From the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland)

Numerous efforts have been made to offer an explanation for photodynamic action. The most recent is that of Blum (1930), who obtained hemolysis of red blood cells with a previously irradiated solution of eosin, and sought to explain it by demonstrating the production of a peroxide during the irradiation. Later Blum and Spealman (1933) were unable to substantiate this by further experiment.

During my work on the effect of photodynamic action on normal and malignant cells, I began to suspect that possibly the product of irradiation of these dyes as described by Wood (1922) might be in some way responsible for this action. With this in mind the following method was devised for the making and the isolation of the photocompound of fluorescein.

A photocompound of fluorescein was made by continuous irradiation of a dilute solution of sodium fluorescein in distilled water; 50 milligrams of the dye were dissolved in a two-liter Erlenmeyer flask of distilled water. Rays of sunlight were concentrated to a point focus on the flask by means of a 4½-inch reading glass. At first the solution fluoresces brilliantly, but slowly diminished until after some 300 hours of irradiation the fluorescence of the solution completely disappears, leaving a product which Wood calls the photocompound of fluorescein or photofluorescein. Wood described this compound as being an intermediate product in the bleaching reaction of the dye, and states that further continued irradiation will result in bleaching.

In order to reclaim the photocompound from this very dilute solution, it was precipitated by the addition of a small amount of hydrochloric acid and separated by filtering. After thorough washing with distilled water the precipitated dye was made back into its sodium salt by the careful addition of barely enough sodium hydroxide to carry it into solution. Then by careful evaporation the crystalline material was obtained, weighed, and dissolved in Locke's solution in quantity such that 0.1 cc. contained one milligram of photofluorescein. Glass electrode pH determination to check the possibility of excess alkali was

[1] Aided by a grant from the International Cancer Research Foundation to Dr. W. H. Lewis.

made on a 1/1,000 dilution in Locke's solution. The pH was found to be 7.85.

Red blood cells were obtained from normal healthy rats. Heparin was used to prevent clotting. The plasma was removed after centrifugation and the cells washed thoroughly with Locke's solution. Various alkaline buffer solutions were used in control experiments to rule out any effect of the slight alkaline reaction of the photocompound. All experiments were carried out in a water bath in which the temperature was controlled at 38° C.

Nine series of experiments were set up to test the hemolytic action of this photocompound and to provide the necessary control tests.

Series 1. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution containing 1.0 milligram of photofluorescein. This was incubated in the dark for two hours at 38° C. Complete hemolysis resulted.

Series 2. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution containing 1.0 milligram of (ordinary) fluorescein. This was incubated in the dark for two hours at 38° C. No hemolysis resulted.

Series 3. Same as Series 2 except that this was incubated in the presence of strong sunlight for 2 hours at 38° C. Complete hemolysis resulted.

Series 4. One cc. of 1 per cent red blood cells in Locke's solution was centrifuged and the Locke's solution drawn off. To the remaining red blood cells was added 2 cc. of Locke's solution containing 1.0 milligram of fluorescein which had been previously irradiated with sunlight for 2 hours. The resulting combination of red blood cells and the previously irradiated dye in Locke's solution was then incubated in the dark for 2 hours at 38° C. Partial hemolysis resulted.

Series 5. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution buffered to pH 8.0. This was incubated in the dark for 2 hours at 38° C. No hemolysis resulted.

Series 6. Same as Series 5 except that the 1.0 cc. of Locke's solution added was buffered to pH 9.0. This was incubated in the dark for 2 hours at 38° C. No hemolysis resulted.

Series 7. Same as Series 5 except that the 1.0 cc. of Locke's solution added was buffered to pH 10.0. This was incubated in the dark for 2 hours at 38° C. Slight hemolysis resulted.

Series 8. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution. This was incubated in the dark for 2 hours at 38° C. No hemolysis resulted.

Series 9. Same as Series 8 except that this was incubated in the

presence of strong sunlight for 2 hours at 38° C. No hemolysis resulted.

From the results given it can be seen that photofluorescein is capable of producing hemolysis in the dark to the same extent that fluorescein is capable in the light, and that the unirradiated fluorescein has no apparent hemolytic action in the dark. Also, that a short two-hour previous irradiation of a solution of the dye produces enough of the photocompound to produce a partial but well marked hemolysis. The buffer solution controls showed no hemolysis except in the case of pH 10.0, in which slight hemolysis occurred. This result rules out any possible effect of the pH 7.85 reaction of the photofluorescein. By a series of trials it was found that approximately two hours in the dark were required for a 1:2,000 dilution of the photofluorescein to hemolyze one cc. of 1 per cent red blood cells.

It is logical to suppose that although long periods of irradiation are necessary for the formation of appreciable quantities of the photocompound, shorter periods of irradiation will produce sufficient amounts to produce hemolysis when the cells are in contact with the dye during the irradiation, or when the dye is previously irradiated. Just what changes are induced in the molecule by the irradiation are as yet unknown. Wood has shown that the absorption spectrum of the photofluorescein has shifted toward the red end of the spectrum.

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THE VALIDITY OF THE CENTRIFUGE METHOD FOR ESTIMATING AGGREGATE CELL VOLUME IN SUSPENSIONS OF THE EGG OF THE SEA-URCHIN, ARBACIA PUNCTULATA

HERBERT SHAPIRO[1]

(From the Physiological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

A problem which frequently arises in biological work is the determination of the total volume of the cells whose functional activities are being studied. This appears, for example, in measurements of oxygen consumption (Whitaker, 1933; Gerard and Rubinstein, 1934), where it may be desirable to give absolute, rather than relative figures. With free cells whose outline closely approximates that of a sphere, such as certain marine eggs, it is possible to estimate the total volume of material to a degree of precision depending upon the regularity of configuration of the particular species of cell, by measuring the diameter of the cells, and the concentration of cells with a suitable counting chamber. This procedure cannot be followed when cells are of irregular outline, for then the volume of the individual cell is not easily computed; one must then resort to a method such as centrifugalization. The problem then becomes one of ascertaining how closely the volume of the packed cells, as estimated from the length of the capillary occupied by the mass of material, approaches the true volume, and determining the complicating effect of extracellular structures such as enveloping membranes, or other layers of varying dimensions. A special case is considered here, viz., that of the egg of the Woods Hole sea-urchin, *Arbacia punctulata*. A justification for a detailed inquiry of a largely routine nature lies in the wide use of these cells for physiological work. Harvey (1932) has collocated the quantitative data pertaining to the various aspects of the chemical and physical properties of this cell. No validation of the centrifuge method has appeared, although numerous investigators have used it for this and other cells to determine egg volume. The *Arbacia* egg is good material for this study because, owing to its negligible departure from perfect sphericity of shape, its volume can be computed fairly precisely from a measurement of the egg diameter; and the material is obtainable in abundance. To consider the limits of error in the use of the centrifuge,

[1] National Research Council Fellow in the Biological Sciences.

under the conditions specified, for a measure of the aggregate volume of cell populations, the evidence will be given under these general headings: methods of measuring concentrations and volume (hemacytometer and centrifuge), distribution of egg sizes, the stacking of cells, extracellular structures, the general effects of centrifugalization on the packing of cells, and on their subsequent viability.

Relative Advantages of Counting and Centrifuging

In weighing the relative advantages of the two methods it is to be noted that the centrifuge method cannot be used where the volume of cells must be known before they are used, or where it is desired to follow the effect of experimentally imposed conditions upon embryological development after measurements have been made. The hemacytometer method, on the other hand, although slightly more laborious, and involving measurement of cell diameters and computation of their volumes, requires only small samples of material, and can thus be used without reference to considerations of quantity of material available.

Distribution of Cell Volumes

The measurement of the egg volume was the most precise datum available, and was computed, after determination of the diameter, from the formula,

$$V = 4/3\pi r^3.$$

Since volume is a cubic function of the radius, r,

$$dV/dr = 4\pi r^2,$$

and it may be noted that the difference in volume accruing from an error of 1 μ in the measurement of the diameter of a 74 μ egg is about 4 per cent; and that the radius is to be determined as accurately as possible.

A specially designed glass chamber, about 0.6 mm. deep, and with optically plane walls, was used to contain the eggs. After a suspension was pipetted into it, the open portion was covered over with a cover slip, and evaporation thus prevented. Egg diameters were measured with a filar ocular micrometer whose scale could be read to a fraction of a micron. Recalibrations of the ocular scale were frequent, to remain certain of its constancy. From each batch of eggs used, the diameters of about ten eggs were measured, and their volumes averaged, to secure a representative figure.

As regards the extent of dispersion of egg volumes, it can be said from inspection of Fig. 1 that the volumes of eggs from different

females vary considerably. It is not feasible, then, to assume a diameter of 74 μ (a figure commonly employed) when the variation is so great. The curve was drawn from data on the volume and diameter of eggs counted and measured throughout the summer of 1934. The mode occurred at slightly less than 72 μ diameter, corresponding to a volume of about 193,000 μ^3. The extremes encountered in these measurements were diameters of 64 and 81 μ. Whether the consider-

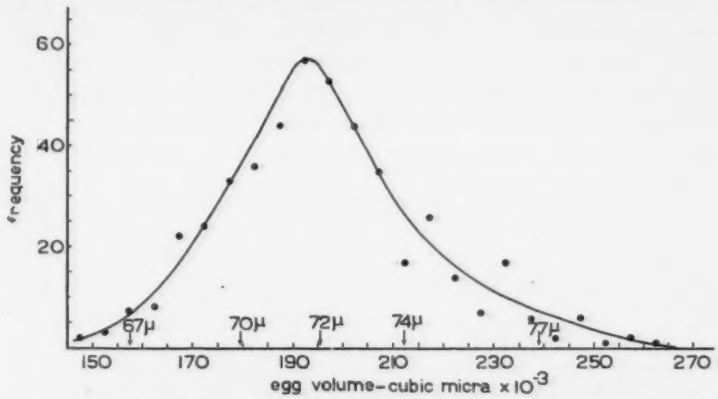

FIG. 1. Distribution of volumes of 467 eggs from 45 urchins selected at random during the season of 1934. The mode occurred at an egg diameter of slightly less than 72 micra.

ably wider distribution and different mode for *Arbacia* eggs reported by Glaser (1914) is intrinsically a seasonal effect and may obtain at other breeding periods, remains to be determined. Not only is the absolute diameter less variable for individual females, but the degree of dispersion of diameters of cells from one animal is approximately constant, regardless of the mode. To determine how individual urchins compare in the variation of egg volumes, 9 eggs were selected at random from each of 14 animals, and the cell volumes measured. The total variation in egg volume was similar to that of Fig. 1, but the coefficient of variation ($v = 100\sigma/M$, σ = standard deviation) was approximately the same for the eggs from each urchin; the average coefficient of variation was 5.4 per cent.

Theoretical Considerations: the Stacking of Cells

If rigid spheres be packed with their centers in line with each other, they occupy 52.36 per cent of the volume of the container; when nested fully, 74 per cent. These values hold regardless of the absolute diameter of the spheres, provided they are all of the same size. Thus,

if *Arbacia* eggs were allowed to settle unhampered in a capillary tube, the volume read off should be 135.2 per cent of the true volume. They are prevented from actually nesting as close as this, under the influence of gravity alone, by the presence of the jelly, which envelops eggs freshly shed or removed from the ovaries. Owing to the presence of the jelly, whose average volume (from 15 cells) in one batch of freshly shed eggs was 1,123,000 μ^3 per egg, the eggs packed by gravity alone would occupy a volume equal to 9.9 times that of the eggs alone if the jelly envelope were to act as a rigid sphere. This ratio would be still higher (14.2 times) if we were to consider eggs which were taken after they had been allowed to remain in sea water for several hours, and whose jelly had consequently swollen to some degree.

Observations on the Jelly

Before they were counted, most of the eggs had large jelly envelopes; about 5 per cent had small coats of jelly. When centrifuged lightly (20 × gravity) in a test tube for one minute (to precipitate the eggs) the cells lose some of the outer looser layer of the jelly shell. In most of the batches of eggs used, even before handling, about 10 per cent of the eggs had little or no jelly. After pouring one batch of eggs back and forth between two tumblers, about nine times, about 50 per cent of the cells lost their jelly. Before sample drops were taken for counting, the eggs were thoroughly stirred in the tall Stender dish into which they had been poured originally. After about ten repeated resuspensions occasioned by withdrawal of samples for counting, about 50 per cent of the eggs were observed to have little or no jelly, and from the remainder the jelly was mostly sloughed off. The gradual reduction of the jelly envelope could be observed by examination of samples of the suspensions, between counts, in Chinese ink. To determine the amount of swelling which the jelly undergoes, the diameters of eggs and of jelly of cells freshly placed in a suspension of Chinese ink in sea water at 8:33 P.M. (urchin opened at 8:27 and eggs placed in dry Syracuse watch glass) were measured (8:35–8:44 P.M.). The average volume of the jelly of 15 cells was 1,123,400 μ^3 per cell. The same cells were measured again about two hours later (10:45–11:00 P.M.), and the average volume of the jelly was found to be 1,690,000 μ^3, or an increase, due to swelling, of about 50 per cent. When considered individually, the jelly of each cell, without exception, displayed the increase.

In centrifuging in dilute suspensions in wide tubes, and in an isosmotic and isopycnotic medium, the jelly is drawn over toward the centripetal end of the egg. When a column of eggs packed in a capillary tube was examined after centrifuging, the centripetal end of the

column frequently showed a transparent region of varying length (1–1.5 mm.); this was taken to be jelly which had found its way to that region of the tube under the influence of centripetal force.

Comparison of Hemacytometers

Since the hemacytometer is an instrument originally designed for use with much smaller cells than *Arbacia* eggs and since, further, the diameter of the egg with its jelly usually greatly exceeds the depth of the 0.1-mm. slide, it was desirable to compare the latter instrument with one of twice its depth (0.2 mm.) and hence larger than the dimensions of the extracellular structures. For estimating the concentration of suspensions with hemacytometers, each batch of eggs was divided into three lots of the same concentration: *A*, for counting with hemacytometer of 0.1-mm. depth, *B*, for counting with 0.2-mm. hemacytometer, *C*, for centrifuging without having been subjected to preliminary counting, which entailed removal of the jelly in various degrees. As a matter of routine, some eggs were inseminated to determine their fertilizability. Only batches with a high percentage of fertilization (95 or more) were used. To remove large particles, the eggs were passed before counting and centrifuging through bolting silk, the size of whose mesh was approximately 175 μ on a side. In consequence of this treatment, about 10 per cent of the eggs were observed to have their jelly much reduced, or absent. The hemacytometers and cover slips were cleaned and dried thoroughly before each count was made. Before each sample was taken, the eggs were suspended uniformly, the suspension taken up quickly in a pipette, with the coverslip held in readiness by a pair of forceps, and a sample drop placed rapidly over the rulings, and the cover slid on immediately. The procedure usually resulted in a fairly even distribution of the cells. Care was taken to avoid irregularity in distribution of eggs in the hemacytometer. Only those drops were counted wherein the eggs appeared uniformly distributed after the cover glass was placed on the counting chamber. Counts on Lot *A* were made alternately with counts on Lot *B*. The 0.1 hemacytometer was ruled so as to contain four large squares (each 1 mm.^{2}) and the 0.2 instrument was ruled into 16 squares (1 mm.^{2}), hence more sample drops were counted, as a rule, with the shallower instrument. After counts were made by the above procedure, the concentration of Lot *A* was measured with the 0.2-mm. chamber, and commutatively, the 0.1-mm. chamber was used for Lot B. These latter measurements were fewer in number than those of the main series. The results, which are given in Table I, show for most counts, ap-

proximately a 12 per cent increase in the concentration when measured by the deeper slide. This is attributed to the influence of the jelly, and the action of the coverslip in tending to "squeeze out" eggs when placed over the drop on the shallower trough. In comparison with the centrifuge determinations, to be described presently, the results with hemacytometers were not found to be as reproducible. Averaging hemacytometer counts yielded a fair approximation to reproducible figures.

TABLE I

Comparison of concentration of egg suspensions as estimated by shallow hemacytometer (0.1 mm.) and by deeper hemacytometer (0.2 mm.).

Date	No. sample drops	Lot *A* 0.1 hcyt. *eggs per cc.*	No. sample drops	Lot *B* 0.2 hcyt. *eggs per cc.*	Difference in per cent *B/A*	No. sample drops	Count of Lot *A* with 0.2 hcyt.	No. sample drops	Count of Lot *B* with 0.1 hcyt.	Difference in per cent *A/B*
August 25....	9	205,000				5	220,200			
29....	9	391,500	6	394,600	+ 0.8	1	343,500	2	306,000	+12.2
30....	11	192,000	5	231,500	+20.6	2	192,300	2	200,000	− 3.8
September 3.	6	167,400	3	189,500	+13.2	2	251,800	1	158,000	+59.4
6.	9	237,000	3	265,200	+11.9	1	286,500	3	260,000	+10.2
10.	6	179,000	3	201,000	+12.3	2	251,000	4	210,300	+19.3
19.	10	178,500	6	174,400	−2.35					

Centrifuge Tubes and Centrifuges

Centrifuge tubes of the type diagrammed in Fig. 2, with a capillary length which varied from 6 to 7 cm., were used. The tubes were sealed off at one end, to avoid possible loss of a slight amount of fluid at high centrifugal forces, as may occur in the use of tubes of conventional hematocrit design, with open ends. Inasmuch as sealing the tubes at one end in this way entails the formation of a meniscus, the closed portion of the capillary was blown out very slightly in an attempt to compensate. The error involved is about 0.5 mm., and in a column of 6 cm. this is a volumetric error of only a fraction of 1 per cent. Krueger (1930) placed a small drop of mercury in the bottom of the tube to secure a more nearly plane column end of cells. Hastings (1921) described the use of a graduated thermometer capillary as a hematocrit, designed by E. L. Scott. Ungraduated capillary tubes of bore 0.8 mm. and 1.6 mm. were used in the present experiments, and gave comparable results at 2,700 and 7,700 × gravity. The capillaries were calibrated for volume per unit length by filling with mercury to various lengths, and weighing precisely.

The cells were mixed and suspended by gentle agitation. A measured sample was pipetted up, and the suspension of cells allowed to flow quickly from the pipette (to avoid settling) into the wide tubing spliced to the capillary. It is not necessary initially to fill the capillary before centrifuging for the air is displaced by the suspension during centrifuging, and the cells and the liquid fill the capillary in a continuous column. The capillary can be cleaned out easily by inserting into it a capillary pipette attached to a tap water suction pump, and immersing the mouth of the tube under water.

The length of the column was measured by placing the tube on a mirror (to avoid parallax) parallel to a steel rule graduated into millimeters. In this way the length of the column could be estimated to about 0.2 mm. The agreement between duplicate determinations of total egg volume by centrifuging was good, and, in the writer's hands, the centrifuge gave less variable results than the hemacytometer. The chief variable in readings of this nature seems to arise from the inhomo-

FIG. 2. Diagram of centrifuge tube, to show capillary and cup for receiving cell suspension. Used for centrifuging at 2,700 times gravity.

geneity in the distribution of the eggs in the suspension from which samples are pipetted. Seventeen duplicate determinations of volume by centrifuging at 2,700 × gravity showed an average agreement of 2.4 per cent, which was much better than duplicate determinations by successive hemacytometer counts. The centrifuge used was a type generally available (International, size 2, head 325); the centrifugal force attainable with this machine was 2,700 × gravity, and large tubes of any size could be accomodated. The centrifuge method may be used in two ways: (*a*) for ultimate packing; or (*b*) where a high speed centrifuge allowing nearly complete packing is not available, the introduction of a conversion factor, when the relative centrifugal force and duration of centrifuging are maintained constant.

General Effects of Centrifuging Cells

The first run was made on material whose measurements are given in Fig. 3. It is clear that for any given mass of cells the degree of packing of the cells depends upon two variables, viz., the duration of centrifuging, and the centrifugal force. Each of the component curves

becomes asymptotic to a given capillary volume at a particular centrifugal force. In this sense, perhaps, constant volumes are not obtained, but if centrifuged long enough, the volume is constant within the errors of measurement. At an early stage of packing, it is relatively easy, at low centrifugal forces, to compress the egg suspension to a smaller volume. At any specified centrifugal force a final degree of packing of cells is attained after a minimum amount of centrifuging. In this

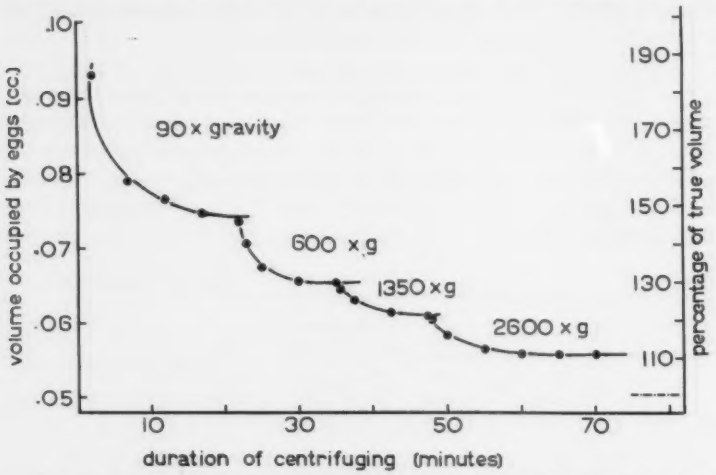

FIG. 3. Effect of duration of centrifuging, and centrifugal force on the volume occupied by a suspension of cells in a capillary. All the data were obtained from a single suspension of cells centrifuged in the same tube. The volumes were measured at various times after starting the centrifuge, and when an approximately constant value had been attained, the centrifugal force was raised. At any given centrifugal force the volume decreases rapidly at first and then approaches asymptotically a constant value. The dot-dash line near the bottom of Figs. 3 and 4 represents in each case the "true volume" as determined from hemacytometer measurements. It is to be emphasized that these curves were not intended to be used as a basis for standard conversion factors at centrifugal forces lower than 2,700 times gravity; they are presented largely to represent the nature of the changes which occur. Figs. 3, 4, and 5 were drawn from the same set of data.

particular run, the total "centrifuge volume" of eggs contained in 1 cc. of suspension (0.0559 cc.), as estimated from the volume of the capillary occupied by the packed eggs, was about 10.6 per cent higher than that computed from the hemacytometer (0.0506 cc.). The ratio of 1.8 to 1 of cell volume by centrifuge and hemacytometer, reported by Gerard and Rubinstein (1934), is understandable when it is noted that low centrifugal forces (400 and 750 times gravity) were used, and relatively short periods of centrifuging (1 to 10 minutes). The further analysis of

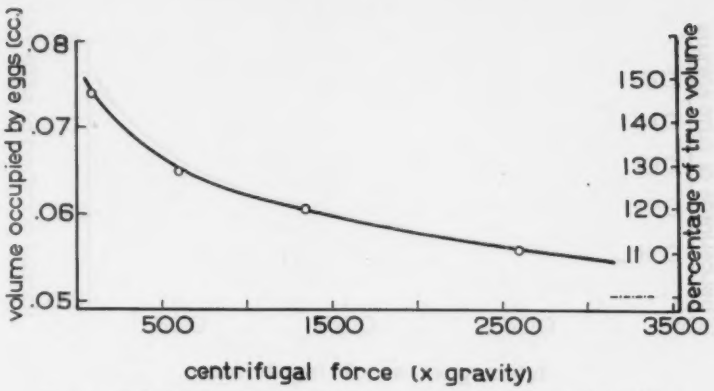

FIG. 4. Relation between centrifugal force and final (asymptotic) value.

this set of data is given in the remaining figures. In Fig. 4, the asymptotic values are plotted for each centrifugal force, and from Fig. 5 it appears that for progressively equal decrements of the excess volume, as read from the capillary length, it was required to increase the centrif-

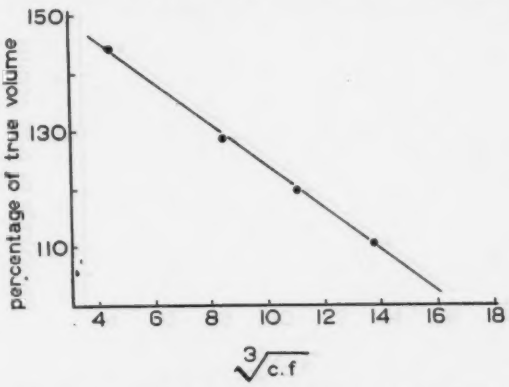

FIG. 5. To show the nature of the dependence of the asymptotic volume occupied by *Arbacia* eggs in a capillary, upon centrifugal force (C. F.).

ugal force, not in equally proportional increments, but rather in cubes of these increments. The expression for this rectilinear relation,

$$P = -3.68\ (C.F.)^{1/3} + 160,$$

where P is the percentage of the true volume, and $(C.F.)$ is the centrifugal force (relative to gravity), holds with good approximation for this

TABLE II

Comparison of total volume of unfertilized eggs as determined by hemacytometers, and by centrifuging in capillary tubes at 2,700 × gravity for 20 minutes. Below, data on fertilized eggs, using volume of unfertilized eggs as basis of comparison.

Date	Volume by hemacytometers	Volume by centrifuge (2,700 × g)	Difference
	cc.	*cc.*	*per cent*
August 25	.0766	.0816	+ 6.5
	.0506	.0537	+ 6.2
29	.0880	.0787	−10.5
	.0440	.0416	− 5.5
	.0440	.0404	− 8.1
	.0880	.0752	−14.6
	.0880	.0744	−15.5
30	.0434	.0424	− 2.4
	.0434	.0452	+ 4.1
	.0656	.0647	− 1.4
	.0434	.0436	+ 0.6
September 3	.0371	.0457	+23.2
	.0742	.0906	+22.1
	.0742	.0925	+24.5
6	.1068	.1242	+16.3
	.0534	.0624	+16.9
	.03204	.03687	+15.1
	.1068	.1238	+16.0
10	.1317	.1289	− 2.1
	.03512	.0373	+ 6.2
19	.0715	.0612	−14.4
	.1072	.0940	−12.3
	.1072	.09514	−11.3
*	.0867	.0940	+ 8.4
*	.0578	.0612	+ 6.0
*	.0867	.09514	+ 9.8
*20	.10632	.1042	− 2.0
*	.0709	.0680	− 4.0
*	.10632	.1028	− 3.4
*	.1122	.1204	+ 7.3
*	.1122	.1185	+ 5.6
	Fertilized Eggs		
August 29	.088	.0763	−13.3
	.088	.0794	− 9.7
30	.0651	.0739	+13.7
September 3	.03902	.04836	+23.8
	.07804	.1000	+28.2

* Volume determined in these cases by dilution method, not hemacytometer.

series of measurements, and affords a satisfactory conception of the nature of the dependence of the "centrifuge volume" upon the centrifugal force. For $P = 100$, i.e., the volume by centrifuge is equal to the true volume (or that calculated by hemacytometer), a centrifugal force of 4,330 × gravity is necessary, as calculated from this empirical formula, assuming that a linear extrapolation can be made. For other sets of data, the curve as a whole would be shifted slightly to the left or to the right, and the extrapolated value would change accordingly.

Results: Agreement between Hemacytometer and Centrifuge Volumes

In Table II, where the data comparing centrifuge and hemacytometer volumes are summarized, a minus sign before the differences indicates that the volume as estimated by centrifuge at the given centrifugal force was less than that as measured by hemacytometer, by the given percentage; and conversely, the plus signs indicate a greater volume by centrifuge as compared to hemacytometer. The values obtained from the two hemacytometers were averaged, and used as a standard basis for comparison with the volumes obtained by centrifuging. A negative value in the comparison of centrifuge with slide would seem to indicate that the slide estimate was too high, or may arise from differences in sampling due to inhomogeneity. The values in the table may be summarized by noting that of 29 determinations at 2,700 × gravity, 15 showed an average of 12 per cent greater volume than that estimated by hemacytometer, and 14 showed an average of 7.7 per cent less than that evaluated by the hemacytometers. Thus the two methods (hemacytometer, 0.1 and 0.2 mm. depths, and centrifuging at 2,700 × gravity for 20 minutes) agreed to within approximately 10 per cent, which may be taken as an average for all the experiments. A few experiments on fertilized eggs are also included in the table.

It would seem that the jelly of the *Arbacia* egg (which, as in these experiments, has been exposed to sea water for approximately one to three hours) is easily displaced or squeezed out by the eggs in centrifuging, and hence does not exert a significant effect in these experiments, for wherever comparisons of Lots *A*, *B*, and *C* were made, where there was no partial reduction of jelly in *C* since it was not mixed and resuspended repeatedly for counting, the measurements of volume by centrifuging resulting from use of portions from each of the lots were identical within experimental error.

Dilution Method

By the use of a procedure suggested by Dr. A. K. Parpart, a check on the two methods described above was made by an independent dilution method. Small portions of egg suspension were diluted to

varying degrees in large volumes of sea water, placed in glass-stoppered bottles, and inverted several times to suspend the eggs uniformly. Then a capillary tube, slightly larger than 1 mm. in bore, and marked off at a length of about 9 cm. (total volume to mark, 0.168 cc.) was quickly immersed and withdrawn, and the total number of eggs contained in the capillary counted directly by examination with a binocular dissecting microscope. Knowing the dilution and the number of cells in the capillary, the original number of cells in the concentrated sample could be calculated. A protocol of some results obtained by this method, which was not studied as thoroughly as the hemacytometer method, is given below. The egg concentration estimated by this dilution method gave figures about 15 per cent lower than those estimated by the hemacytometer.

No. samples counted	Av. concentration by hcytr.	Dilution method	No. eggs per cc.	Centr. tube	$\frac{\text{Centr. vol.}}{\text{Hcyt. vol.}}$	$\frac{\text{Centr. vol.}}{\text{Dilution vol.}}$
10	176,500 cells/cc.	5 cc. : 140	141,520	B	$\frac{.06115}{.07148}=85.6\%$	$\frac{.06115}{.0578}=106\%$
		5 cc. : 500	148,570	D	$\frac{.0940}{.1072}=87.7\%$	$\frac{.0940}{.0867}=108.4\%$
		5 cc. : 1,000	138,700	C	$\frac{.09514}{.1072}=88.7\%$	$\frac{.09514}{.0867}=109.8\%$

In another series of determinations, the dilution and centrifuge methods only were compared, and gave very good agreement. The data are given below; all figures were obtained from measurements on a single suspension.

Comparison of Dilution and Centrifuge Method

No. samples counted	Dilution	Average concentration	Tube	$\frac{\text{Centrif. vol.}}{\text{Dilution vol.}}$
4	2 cc. : 140 cc. s.w.	185,000 cells/cc.	B	$\frac{.068}{.0709}=0.96$
8	2 cc. : 500 " "	184,700 " "	C	$\frac{.1042}{.1063}=0.98$
6	2 cc. : 1,000 " "	167,400 " "	D	$\frac{.1028}{.1063}=0.97$
7	6 cc. : 1,000 " "	177,700 " "		

Data on the Absolute Respiratory Rate of Unfertilized Arbacia Eggs

It may be of interest to note that measurements of the respiration (9/20/34) of eggs taken from urchins kept in laboratory aquaria for a period of about four weeks, with Warburg manometers, at 25.9° C., showed a Q_{O_2} (cu. mm. oxygen per 10 cu. mm. eggs per hour) of 1.03 for unfertilized eggs, and 3.04 for fertilized eggs (average of three determinations of each). The absolute volume of eggs was determined by combining the values obtained by centrifuge and dilution methods. Reduced to 21° C. by applying a Q_{10} of 4.1 for unfertilized eggs (Rubinstein and Gerard, 1934), the Q_{O_2} becomes at that temperature 0.52. More representative is the average of a series of measurements carried out from July to September, 1934, with Warburg and Fenn respirometers, at 25.9° C., and using the hemacytometer method for estimating egg volume (reported in detail elsewhere: Shapiro (1935)). The Q_{O_2} for unfertilized eggs was 1.5. When reduced to 21° C. this value becomes 0.75.

Development of Eggs after Centrifugal Compression in a Capillary

After centrifuging, unfertilized eggs were removed from the capillary and examined microscopically. They were found to be well stratified in the usual manner, drawn out into cylinders, with corners somewhat truncated, a configuration lending itself to more intimate packing of the cells. One batch of cells which had been centrifuged 20 minutes at 2,700 × gravity was left in the capillary 24 minutes in the packed condition, and then removed and inseminated. They retained their elongated form after fertilization, and proceeded to cleave and develop. The pigment remained at one pole of the cell. This was demonstrable repeatedly and indicates that cells can easily survive these experimentally induced conditions. The toughness of the fertilization membrane prevents eggs from elongating, when centrifuged after insemination. The fertilized eggs, after packing, were poorly stratified and in some cases practically unstratified, and polygonal in outline, and retained the fertilization membrane, which usually enveloped the cell closely. They tended to resume the spherical configuration when replaced in sea water after centrifuging. On several occasions it was found that cells fertilized and then packed in a capillary by centrifuging at 7,700 × gravity for 20 minutes would upon removal from the tube proceed to develop as far as free-swimming plutei. Whether water is actually abstracted from the cell as a result of centrifugal compression, is an open question. R. S. Lillie has observed (1918) that fertilized cells, which have shrunken in hypertonic sea water faster than resting egg cells, appear denser than unfertilized

eggs exposed for the same period of time in that they sink faster to the bottom of the container.

Related Experiments at Higher Centrifugal Force

Numerous experiments were made on living eggs swollen and shrunken, and dead cells of various volumes fixed in formalin and centrifuged at 7,700 × gravity to determine volume. The technique involved the use of large volumes of fluids and subsequent decantation of the supernatant fluid, following light centrifuging, in order to get the cells into the small tubes used, which entailed their addition in several small portions, with centrifuging between additions. This procedure was necessary because of the short length of the slots in the high speed centrifuge head, and the use of a long capillary column led to the sacrifice of the wide tube at the upper end, which might receive all the cells at once. The cells were added in small quantities, and the supernatant fluid removed after each preliminary centrifuging to make way for the rest of the cells. A slight (but indeterminate) loss of cells occurred owing to the adhesion of some of the material to the walls of the large test tubes in which they were kept during swelling or shrinking. Thus it was difficult for these reasons to demonstrate the adequacy of the centrifuge method for such cells. Data for normal, living unfertilized and fertilized eggs centrifuged for 20 minutes at 7,700 × gravity were also obtained, and may be summarized by stating that of 15 determinations, 6 showed an average volume 10.6 per cent less and 9 an average volume 12.8 per cent greater than that estimated by hemacytometers. It is of interest to add that when eggs are placed in successive small lots in these small tubes, they appear rhythmically stratified, i.e. each lot shows a light brown layer at the centripetal end.

Summary

Observations of the aggregate volume of *Arbacia* eggs, when centrifuged in capillary tubes, as a function of centrifugal force, and of duration of centrifuging were made. Data on the comparisons of such total cell volume contained in suspensions of *Arbacia* eggs as evaluated by three methods (counting in hemacytometers, centrifuging at 2,700 and 7,700 × gravity, and direct counts of diluted suspensions) are given. It is submitted that the centrifuge method is reliable, to within approximately 10 per cent, for estimation of the total volume of cells in a suspension of unfertilized eggs of *Arbacia punctulata* in sea water provided that it be used with the necessary force and duration for sufficient packing of the cells.

Cells so centrifuged are in the living state, and remain viable, for

upon removal from the capillary, they will proceed to cleave and to undergo considerable embryological development.

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AN ANALYSIS OF THE ACTION OF LITHIUM ON SEA URCHIN DEVELOPMENT

JOHN RUNNSTRÖM [1]

(From the Marine Biological Laboratory, Woods Hole, Mass.)

The fact that lithium added to sea water profoundly modifies the sea urchin development was discovered by Herbst (1892, 1893, 1895). The most important change involved is the enlargement of the entomesoderm at the expense of the ectoderm. It is important to stress that this shift in the relative amounts of ectoderm and entoderm is not occasioned by any destruction or throwing off of ectoderm material during the early development but by some kind of a change in the determination of the egg. This would mean that the potentialities for producing ecto- and entoderm are so affected by lithium as to favor those which are concerned in the production of entoderm. While Herbst (1893) and Spek (1918) in their interpretation of the "lithium" development directed their main attention towards the vegetative part, MacArthur (1924) and Runnström (1928, 1929, 1933) have emphasized the effect of lithium on the animal part of the larva. All our experimental evidence indicates that the susceptibility to the action of lithium is highest at the animal pole and decreases gradually.

The writer (1929, 1933) has developed the conception that in the sea urchin egg, there exist two opposite gradient systems, one preponderating at the animal, the other at the vegetative pole, as illustrated diagrammatically in Fig. 1. According to this hypothesis, the determination along the egg axis would depend upon the balancing effect between the animal and the vegetative gradient system. The lithium type of development would therefore be due to a shift in the normal balance, as illustrated by Diagram *B* in the figure. The animal gradient system weakens and the vegetative gradient system gains the ascendancy. The ring of skeleton-forming mesenchyme cells appears further towards the animal pole and the boundary between ectoderm and endoderm moves closer to the animal pole of the egg. In extreme cases this condition may be carried to the extent that the entire embryo is transformed into entomesoderm.

Under other conditions the opposite effect is known to occur. For example, by exposing the eggs to certain agents, the vegetative gradient system weakens in comparison to the animal gradient system, cf. Dia-

[1] Fellow, Rockefeller Foundation, 1933–34.

gram *C* in the figure. As a consequence of the resulting unbalance, the animal region becomes increased (Herbst, 1904; Lindahl, 1933).

The theory briefly given here is substantiated also by numerous isolation and transplantation experiments carried out by Hörstadius (1935). Only one of these experiments may be mentioned. In the 64-cell stage Hörstadius isolated that one of the two cell rings, originating by the division of the macromeres, which lies at the more vegetative end. This cellular material in normal development gives rise to entoderm only. The isolated ring developed into a larva with both entoderm and ectoderm. Evidently by a regulative process, an almost normal balance had become established in the isolated cell ring. By implantation into this cell ring of one, two, or four macromeres (the most vegetative material), this balance was disturbed and larvæ developed which most strikingly resembled lithium-treated larvæ. Hence an increase in amount of micromere material produced the same effect as an increase in lithium concentration.

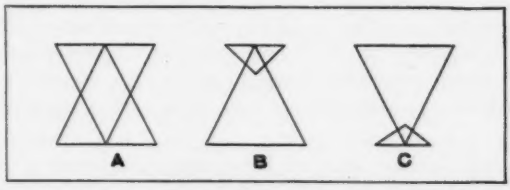

FIG. 1. Diagrammatic representation of the gradient systems in the sea-urchin egg.

The mode of action of lithium has been assumed by Spek (1918) to be a precipitating and swelling effect on the surface of the vegetative cells. This, according to Spek, would account for the "exogastrulation" which is one of the most characteristic effects of treatment with stronger concentrations of lithium. Runnström believes, on the other hand, that such a surface effect is secondary to a more important internal effect and he has experimental evidence to show that lithium penetrates the cells. With dark ground illumination the internal structure of the cells of the "lithium" larva has a coarsened appearance. He assumes that some action of the lithium which is related to this structural change has an effect on the determination and differentiation of the animal pole greater than on the vegetative pole.

Runnström (1933) found that lithium, in concentrations which in themselves are too low to affect development, can be made very active if the eggs, during exposure, are kept in an atmosphere of carbon monoxide containing 5 per cent oxygen. This effect of carbon monoxide is abolished in strong light. The same, as is well known, holds true

from Warburg's work (1932) on the effect of carbon monoxide on respiration. This evidence, that the effects of lithium and carbon monoxide are additive, suggests that these two agencies have a similar action on the developing egg. In support of this is the finding of Lindahl (1933, 1934) that lithium has an inhibiting action on the respiration of the egg. Warburg (1915) has shown that a steady increase of respiration takes place during the early development of the sea urchin egg. It is only this increasing fraction of the respiration which is affected by lithium according to Lindahl. This fraction has a special character and may be identical with the oxidation of breakdown products of certain carbohydrates (Lindahl, 1933, 1934, 1935).

During my sojourn at the Marine Biological Laboratory at Woods Hole during the summer of 1934, I had the opportunity of securing the assistance of Professor Robert Chambers in a microdissection study on the physical properties of the eggs of the sand dollar, *Echinarachnius parma,* treated with lithium.

The eggs of this species proved to be extremely sensitive to the action of lithium. A solution of 100 cc. sea water and 5 cc. of 2.6 per cent LiCl killed them in the early stages of segmentation, while the eggs of *Arbacia punctulata* (similar to the European *Paracentrotus lividus*) segment and develop in this solution for more than twenty hours.

It was found that the sand dollar eggs will develop in a weaker solution, for example: eggs immediately after fertilization were placed in 2 cc. of 2.6 per cent LiCl to 100 cc. sea water and, after a sojourn of 20 hours, were transferred to normal sea water. The larvæ exhibited the typical "lithium" effect, namely, a retarded development, a shift of the position of the mesenchyme cell ring and an enlargement of the entodermal region followed by exogastrulation and differentiation of ectoderm and skeleton.

It was found that the physical state of the cells of the larvæ which had been exposed to lithium in sea water was demonstrably different from that of controls developed in sea water alone. The blastulæ were pierced and held in place in the hanging drop (in the microdissecting chamber) by means of one micro-needle and the epithelial wall was torn by means of another needle. In this way strands of the epithelial cells could be stretched. The cells of the controls, after being torn apart and released, quickly became spherical. On the contrary, the cells from the "lithium" blastulæ remained distorted and spindle-shaped. The difference was striking and left no doubt that a profound change in the state of the cytoplasm had taken place as a consequence of the action of the lithium.

It was also found that the hyaline plasma layer which invests the larva is softened and rendered far more pliable when treated with lithium than the layer investing the untreated larva. This fact nullifies any assumption that an increased resistance to the swelling of the blastocœl and the enlargement of the blastula might have been due to a stiffened hyaline plasma layer.

A further observation on control larvæ may be mentioned in this connection. It is easy to observe, in the late blastula or the early gastrula stage, that the hyaline plasma layer presents many folds on the surface of the most vegetative part of the larva. A microneedle could be inserted into this part of the hyaline layer. When the layer was stretched the folds disappeared and a cone-like process was formed. Evidently the folds are formed when the form of the underlying cells changes preparatory to the invagination of cells during gastrulation. The later fate of these folded parts of the hyaline layer is difficult to follow but it is probable that an unfolding takes place when the entoderm cells subsequently enlarge their surface. No studies were made on the presence of folds in the hyaline layer in lithium-treated larvæ, but the folding of the hyaline layer occasionally was observed also in "lithium" larvæ. This speaks also against a stiffening of the hyaline plasma layer under the action of lithium.

The hyaline plasma layer can still be demonstrated even in the normal pluteus. A striking method is to leave the larva in a dish infested with certain Protozoa. These penetrate the dead larva and consume the cellular portion, leaving intact only the skeleton and an investing, fairly soft membrane.

During the development of the larva a considerable growth of the hyaline layer must also take place. This growth must be due to a secreting activity of the cells which normally are kept together chiefly by the hyaline layer. Herbst (1900) has discussed the factors involved in the coherence of the epithelial cells of the sea urchin larva. He considered the action of the hyaline layer and also a second factor, which according to Herbst is more important, and which keeps the cells together even when the hyaline layer has been removed. It seems probable, however, that this second factor is more pronounced only in larvæ which have been submitted previously to a treatment with calcium-free sea water.

During the early differentiation of the sea urchin larva the change in shape of the cells plays an important rôle. Already in the blastula stage a flattening of the cells has begun to take place. In the pluteus most of the aboral part of the ectoderm is formed by a flat epithelium. The indications are that the hyaline plasma layer plays only a sub-

ordinate rôle in changing the shape of the cells during development. A retardation in the flattening of the blastula cells is very characteristic for the lithium type of development. The observations made in the course of this work do not favor the idea that the retardation is due to the changed qualities of the hyaline layer. Probably retardation is caused by a " lithium " effect on the internal material of the cells.

The pronounced reaction of *Echinarachnius* eggs to LiCl in comparison with their reaction to chlorides of sodium and potassium are shown in the following experiment. Eggs, recently fertilized, were immersed in solutions of the three chlorides in concentrations approximately isotonic with sea water. In a KCl solution the eggs survived for several hours and underwent cleavage. In NaCl the survival was more limited, for, in the course of an hour, a certain percentage of the eggs underwent cytolysis and the rest at varying times somewhat later. In LiCl all the eggs were destroyed within 10–15 minutes. The surface of the egg burst and a part of the contents flowed into the space under the fertilization membrane while the remainder of the egg shrank somewhat into a clear cytolyzed mass in marked difference to the dark cytolized eggs in NaCl.

The experiments reported in the preceding pages tend to show that lithium has a direct influence on the structure of the cytoplasm. But the possibility is not excluded that the action of lithium, in the range of concentrations producing typical " lithium development," is more indirect. This is rendered probable by the following observations. Eggs of *Echinarachnius* were transferred very soon after fertilization into a solution of 100 cc. sea water containing 5 cc. of 2.6 per cent LiCl and into a similar solution containing 0.5 cc. of 0.1 per cent pyocyanine. In the lithium-sea water the eggs were killed in the two-celled stage but in the lithium-sea water plus pyocyanine a development to the blastula stage took place. In the control the development was normal. This experiment was repeated and varied as to the lithium concentration. It was always found that the addition of pyocyanine caused a marked improvement on the development in lithium-sea water. In a few experiments it was also found that the addition of methylene blue somewhat improves the development in lithium-sea water. From experiments carried out by Friedheim (1931), it is well known that pyocyanine has a promoting action on the respiration of several kinds of cells. Runnström (1935) has shown that this is true also for the fertilized sea-urchin egg. It can thus safely be inferred that pyocyanine acts by inducing oxidation processes which are able to replace, to a certain degree, those suppressed by lithium.

Runnström (1928) found that the addition of KCl to the lithium-sea

water in somewhat more than equimolecular concentration to that of the lithium removes the modifying influence of the lithium on morphogenesis. Lindahl (1933) showed that this addition of KCl also removes the inhibitory action of lithium on respiration. From unpublished experiments of mine on *Arbacia* eggs I have found that potassium is more efficient than pyocyanine on the restitution process.

From the facts reported and discussed above it follows that one has not only to consider a general effect of lithium on the physical state of structure but also a specific effect on the metabolism of the sea urchin egg and embryo. This specific effect may possibly be the primary one. These aspects will be more fully discussed in a paper by Lindahl (1935).

Summary

The eggs of *Echinarachnius parma* are very sensitive to the action of lithium added to sea water.

A concentration of lithium was used which produces the typical "lithium development." The epithelial cells of normal and of lithium-treated blastulæ were stretched by microneedles and released. In normal larvæ the cells round up after release; in the lithium-treated larvæ they remain deformed. The hyaline plasma layer does not stiffen in the lithium-sea water and is not necessary for the flattening of the cells which takes place during the development. The presence of folds of the hyaline plasma layer at the vegetative pole in the late blastula and the early gastrula stage is described.

Pyocyanine, added to the lithium-sea water, counteracts the effect of the lithium and improves development. This and other facts indicate that lithium does not only exert an influence on the structure of the protoplasm but also on the respiration.

I wish to express my sincere thanks to Professor Robert Chambers for his generous assistance in carrying out the micro-dissection work reported in this paper and for correcting and criticizing the manuscript.

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THE LUNGS OF THE MANATEE (TRICHECHUS LATIROSTRIS) COMPARED WITH THOSE OF OTHER AQUATIC MAMMALS

GEORGE B. WISLOCKI

(From the Department of Anatomy, Harvard Medical School, Boston, Massachusetts)

In 1929 I reported the histological structure of the lungs of the porpoise (*Tursiops truncatus*) and discussed the findings in relation to the aquatic mode of life of whales. The present report concerns the finer structure of the lungs of another aquatic mammal, the manatee (*Trichechus latirostris*), belonging to the order of Sirenia or sea cows. Concerning the histology of the sirenian lungs, the only previous account is a description by Pick (1907) of the lungs of *Halicore dugong*, which omits a very complete account of its finer histology.

The ultimate aim of these studies is to obtain a knowledge of the modification of the respiratory tract in mammals which have adopted an aquatic mode of life. To date, in addition to the observations of Pick on the dugong, fuller accounts exist for certain Cetacea, namely, three of the porpoises, *Delphinus* (Fiebiger, 1916, and Lacoste and Baudrimont, 1926), *Tursiops truncatus*, the bottle-nosed porpoise (Wislocki, 1929), and *Phocaena communis*, the harbour porpoise (Lacoste and Baudrimont, 1933). The present account of the lungs of the manatee adds the new world representative of the order Sirenia to the observations of Pick on the old world form, the dugong, and makes it possible to undertake a wider comparison of the Sirenia with the porpoises of the order Cetacea.

Anticipating the results of our examination of *Trichechus*, it may be said that they resemble rather closely the account given for the dugong by Pick. Turning to the Cetacea, comparison of *Delphinus* and *Tursiops* (both Delphininae) has shown that these two species agree with one another in almost every detail. On the other hand, the smaller harbour porpoise, *Phocaena*, representing the Phocaeninae, exhibits, according to Lacoste and Baudrimont, a number of major differences from the Delphininae. The latter finding suggests that there may be a number of modes of specialization of the lungs within the order Cetacea. Finally, a comparison of the two types of Sirenia (which in regard to lung structure show a close resemblance) with the two types encountered thus far in the porpoises, should allow us in a

beginning measure to discriminate between the structural changes which are generalized aquatic adaptations and those which represent specializations within the narrower confines of an order or family of mammals.

Material

The material on which the present description is based consists of the lungs of an adult manatee, freshly fixed *in toto* in 10 per cent formalin. Sections of the lungs were studied after staining with haematoxylin and eosin, Mallory's connective tissue stain, and Weigert's resorcin fuchsin. The preservation of the tissues, although by no means perfect, allows a considerable amount of the histology of the lungs to be ascertained, as the accompanying photographs of the sections illustrate.

Description of Material

The description of the material will be kept as brief as possible, the appended illustrations offering the best means of presenting the findings. No presentation is deemed necessary of the naked-eye topography of the lungs, because on reading over Pick's rather complete account of the gross appearance of the lungs of the dugong and comparing it with my specimen of the manatee, I have come across no salient differences in anatomical arrangement. It will suffice, therefore, to present merely one photograph of the gross appearance of the specimen, namely, a transverse section or rather slab of tissue obtained by cutting in half one of the two elongated, flattened lobes which characterize the sirenian lungs (Fig. 1). To the right of the figure on the ventral border the main bronchus can be seen accompanied by the pulmonary artery (*p. a.*) and vein (*v. p.*).

Explanation of Plate I

Fig. 1. A transverse slab of tissue from the right lung of a manatee. Notice the main bronchus (*br. 1*) on the ventral surface. Nearby are the pulmonary artery (*p. a.*) and vein (*p. v.*), besides a subsidiary bronchus (*br. 2*). × .34.

Fig. 2. A section of the slab shown in the preceding figure covering about two square centimeters, showing the tremendous size of the alveolar sacs and the heavy septa bounding them. × 2¾.

Fig. 3. A section of human lung, at the same magnification as the preceding figure, to show the tremendous differences between the two in size of air sacs, thickness of the pleura, and the density of the stroma of the lungs. × 2¾.

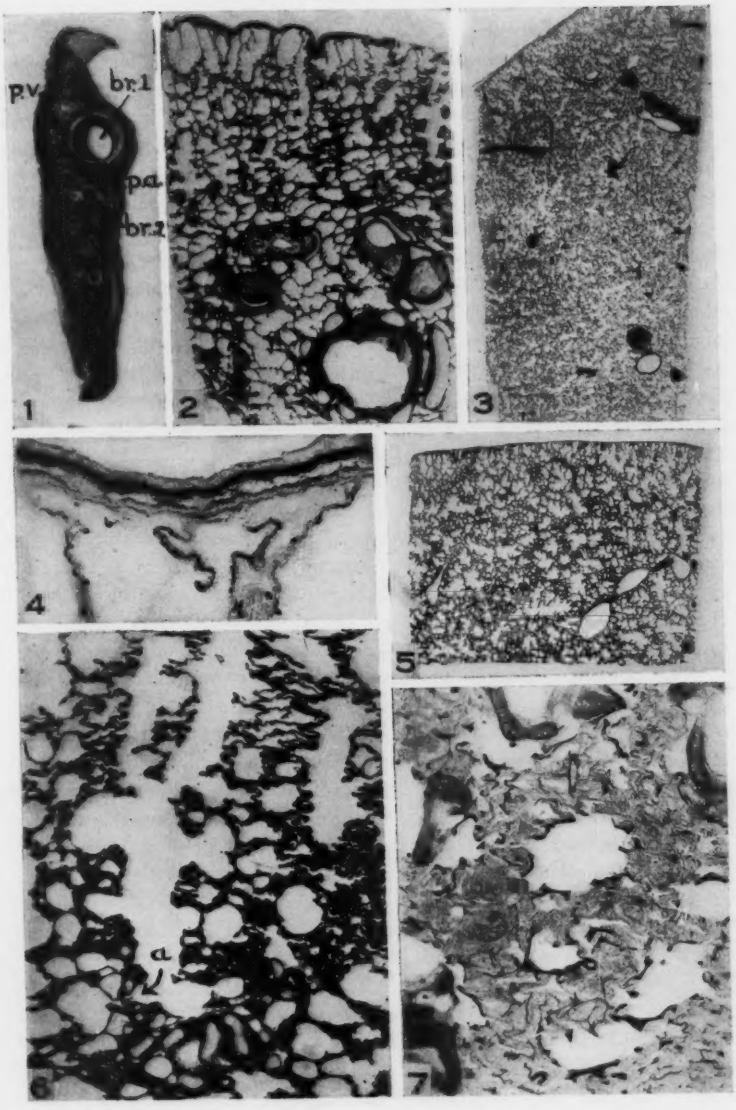
Fig. 4. A photograph of the pleura of the manatee stained for elastic tissue to show: (1) the outer collagenous layer; (2) the dense lamina of elastic tissue in the middle; and (3) on the inner side the vascular layer. × 30.6.

Fig. 5. A section of a cat's lung for comparison with the manatee lung (Fig. 2) and the human lung (Fig. 3), all at the same magnification. × 2¾.

Fig. 6. A photograph showing the configuration of one of the large tubular air sacs from the lung periphery. At (*a*) is indicated the communication of a terminal bronchiole with the air sac. × 11.22.

Fig. 7. A photograph of a porpoise's lung (*Tursiops truncatus*) stained for elastic tissue for comparison with a similar preparation made from the lung of a manatee. × 30.6.

PLATE I



On taking a block representing about half the diameter of the lung out of the slice of tissue shown in Fig. 1 and preparing sections from it, the finer topography of the manatee lung is revealed. Figure 2 represents about two square centimeters of lung tissue shown at low magnification. For comparison with more familiar lung tissue, sections of normal human lung (Fig. 3) and cat lung (Fig. 5) are shown beside it. The tremendous size of the air sacs and the relatively coarse texture of the lung tissue are at once apparent as compared with either man or cat. The air sacs are many times the size of any terrestrial mammal, and the word "giant alveoli" has been applied by Pick to the similarly large ones encountered in *Halicore dugong*. A further characteristic of both the manatee and dugong is the presence of extremely large air sacs at the border of the lung immediately underneath the pleura. These peripheral air sacs, which are clearly shown in Fig. 2, are roughly cylindrical or tubular in shape contrasted with the smaller polygonal ones in the interior which do not reach the surface. The tubular peripheral air sacs may reach a length of six millimeters and a diameter of from one to two millimeters, whereas the largest ones in the interior are never over three millimeters in greatest length. The tubular air sacs are confined solely to the dorsum of the characteristically flattened lung. The alveolar sacs underlying the pleura on the ventral surface are not elongated. This difference of the two surfaces is related perhaps to the distribution of the bronchial tree which is located on the ventral side of the lung, and sends off its short, stout bronchioles towards the dorsum of the lung.

The terminal bronchioles are short and communicate by wide openings with the large alveolar sacs (Figs. 9 and 11). The openings of the terminal bronchioles into the air sacs are pores having a diameter of from 0.6 to 0.3 mm., hence exceedingly large in diameter compared to similar openings in other mammalian lungs. In the porpoise (*Tursiops*) by comparison the bronchioles are much longer and more slender than in the manatee, and the terminal openings are correspondingly smaller (0.1 to 0.2 mm.). The columnar epithelium which lines the bronchial passages terminates abruptly at the site of union of the bronchioles and the air sacs (Figs. 9 and 11), indicating that the division between the essential respiratory tissue and the conducting passages is located here. The cartilaginous armature of the bronchial tree is highly developed, extending into the walls of the smallest bronchioles. Bits of cartilage can be seen in Figs. 9 and 11 surrounding the smallest bronchioles and their openings into the air sacs. The presence of extensive cartilaginous armature in the bronchial tree is characteristic of all aquatic mammals thus far studied,

including the dugong described by Pick. A good description of the arrangement of the cartilage in the trachea and bronchi is given by Pick for the dugong, and, since from my examination of the manatee conditions appear to be the same, I shall give no detailed account of it. Mention should be made of the fact, however, that Pick found calcification present in the larger cartilages of trachea and bronchi. This is not the case in my specimen of the manatee. The individual bits of cartilage in the relatively short, stout terminal bronchioles of the manatee are coarser and more widely separated than in the porpoise (*Tursiops*).

The bronchioles exhibit in their walls a moderate amount of smooth muscle and elastic tissue, nothing like, however, the tremendous amounts of these tissues observed in porpoises by Fiebiger, Lacoste and Baudrimont and myself, where there is a specialized system of myo-elastic sphincters in the terminal bronchioles. Such sphincters are totally lacking in the manatee, as presumably they are also in the dugong, since Pick makes no mention of them. This difference does not discriminate essentially between the sirenians and porpoises, however, for in one of the latter, the small harbour porpoise (*Phocaena*), Lacoste and Baudrimont have not found an elaborated system of sphincters. The elastic tissue in the walls of the bronchioles of the manatee appears predominantly in the form of a lamina of fibers lying between the cartilages and the lumen of the bronchiole. An outer elastic membrane surrounding the cartilages or external to them is not well defined as in *Tursiops*. I have also found in the walls of the bronchioles of the manatee rather extensive accumulations of lymphoid tissue exhibiting here and there "germinal" centers.

The alveolar sacs of the manatee lung, as stated above, are voluminous. They are bounded by excessively heavy septa which, in so far as I can analyze them, appear to be composed of a considerable amount of collagenous fibers, some smooth muscle, and relatively little elastic tissue (Figs. 6, 8, and 10).

The smooth muscle and elastic tissue occurring in the walls of the alveolar sacs of the manatee are not diffusely distributed but bear a definite relationship to the contours of the sacs. It may be seen in any low power field of the manatee lung that the alveolar sacs (both the elongated ones on the periphery, as well as the irregularly shaped ones buried beneath the surface) are subdivided into a series of compartments or lesser sacculations by septal folds (Figs. 6 and 8). These folds have, naturally, free borders which project into the lumen of the alveolar sacs. Moreover, in the tubular air sacs at the surface of the

lung, it can be observed that the folds tend to encircle the elongated sacs. Consequently when a sac is cut longitudinally the free edges of the septal folds appear as a series of small knobs as shown in Fig. 8 (*mus.*). These enlarged edges are localities where smooth muscle and elastic tissue intermingled occur in the form of dense bundles (Figs. 8 (*mus.*) and 10), capable, one would presume, of acting as a series of sphincters which control the size of the air sacs. Figure 10 shows these septa stained with resorcin fuchsin to demonstrate elastic tissue. Figure 7 shows for comparison the lung of a porpoise (*Tursiops*) similarly stained. Characteristic of the latter is the smaller size of the alveolar sacs and the much greater abundance of elastic tissue, which is much more diffusely scattered, although here, too, there is a tendency for the elastic tissue to attain its maximum density along the septal margins.

As in the porpoises which have been examined, the pulmonary capillaries form a double bed in the alveolar walls. Each surface of a septum lying between two adjacent air sacs is supplied by separate capillaries separated from one another by collagenous tissue lying in the center of the septum. This double layer of capillaries, not mentioned in Pick's description of the dugong, was probably overlooked in his material. It appears to be a constant arrangement in the obligate aquatic mammals thus far described (*Delphinus*, *Tursiops*, and *Phocaena*).

No lining epithelium can be made out with certainty in the walls of the air-containing, distended alveolar sacs, but in certain areas of

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EXPLANATION OF PLATE II

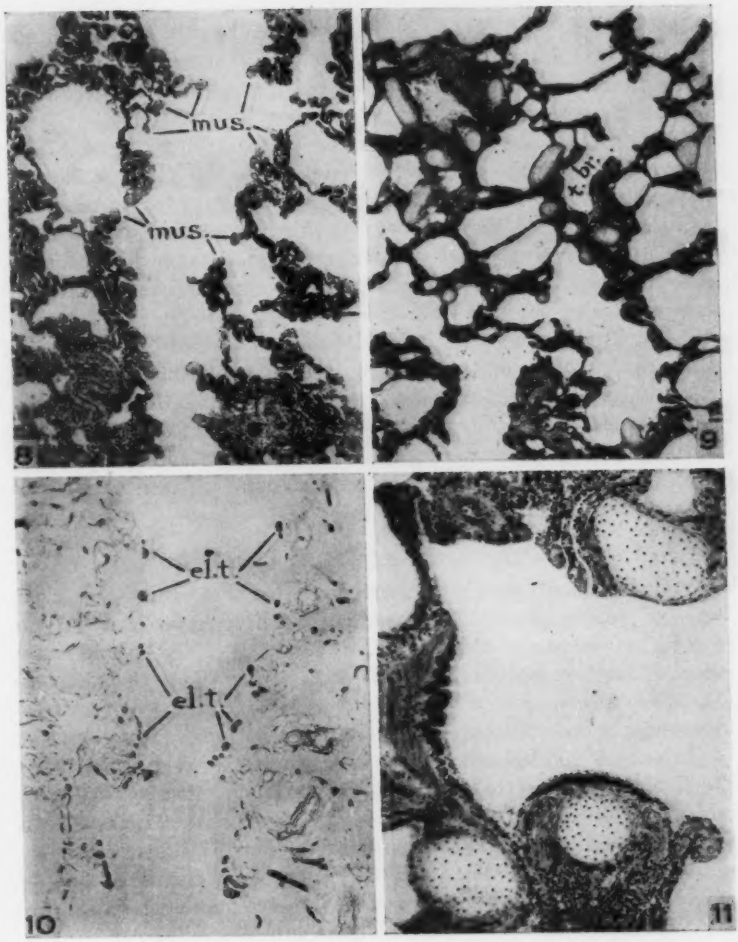
FIG. 8. A photograph of an air sac of the manatee showing the partial subdivision or sacculation of the alveolar sac by stout septa. The free borders of the septa, sometimes slightly club-shaped, are composed of stout bundles of smooth muscle (*mus.*). × 27⅛.

FIG. 9. A photograph showing large air sacs in the interior of the manatee lung showing a communication at one place with a terminal bronchiole (*t. br.*). Numerous bits of cartilage are visible in the walls of the air sacs in this region. × 17.

FIG. 10. A section of manatee lung stained for elastic tissue for comparison with Fig. 8 (both at the same magnification) from which may be gained that the elastic tissue (*el. t.*), similar to the smooth muscle, is condensed at the free borders of the septa which partially subdivide the air sac, the two together forming stout myo-elastic bundles. Further, from comparison of this figure with Fig. 7, taken from a porpoise's lung, both stained for elastic tissue and equally magnified, it may be observed how much more abundant elastic tissue is in the lung of the porpoise than in the manatee. × 27⅛.

FIG. 11. A photograph at the point of communication of a terminal bronchiole with an air sac. On the left and bottom of the picture low columnar or cuboidal epithelium of the terminal bronchiole can be seen. Three bits of cartilaginous armature are visible. × 85.

PLATE II



the tissue, in which the air sacs are atelectatic, a distinct layer of haematoxylin-stained, oval cells, which can be best accounted for as respiratory epithelium, can be observed on the surface of the wall of the alveolus. Similarly the apices of the tubular air sacs beneath the pleura appear in some localities to have undergone partial collapse, giving to the portion of the air sac contiguous to the pleura an almost glandular appearance ascribable to the fact that here also by virtue of the relaxation of the walls a lining of what appears to be epithelial cells has been rendered visible.

The pleura of the manatee is 0.3 to 0.5 mm. thick (Fig. 4). This is thinner than in the porpoise, where it is one-half to one millimeter thick, but on the other hand is materially greater than in terrestrial animals (man, cat, Figs. 3 and 5). Microscopically the pleura is made up of dense bundles of collagenous and elastic fibers besides smooth muscle. It is well vascularized by numerous small arterioles and veins, and contains in addition occasional recognizable lymph vessels. From without in, it is composed of several layers. Outermost, clothed by mesothelial cells, is a layer of collagenous tissue, followed by a heavy lamina of elastic tissue (Fig. 4). Thereupon follows a vascular layer composed of small arteries and veins imbedded in fibrous tissue consisting mostly of white fibers and some smooth muscle bundles. Innermost is a zone of more delicate fibro-elastic tissue containing capillaries.

Discussion and Conclusions

For convenience and brevity in comparing the architecture of the lungs of aquatic mammals thus far known, I have arranged the principal observations in tabular form (Table I). The porpoises and Sirenia have in respect to the histology of the lungs the following characters in common: a very heavy well-vascularized pleura, large alveolar sacs bounded by excessively heavy alveolar walls composed of fibro-elastic and smooth muscle tissue; double layers of capillaries in the alveolar walls, so that single capillaries do not subserve simultaneously the gaseous exchange in two adjacent alveoli; the presence of a well-developed cartilaginous armature extending to the terminations of the smallest bronchioles. For the present these characters may be regarded as the common features of the lungs of mammals which have accommodated themselves to an obligate aquatic existence.

The lungs of the manatee and dugong appear in most respects to be almost identical in structure. They differ specifically from the porpoises in the possession of giant air sacs, with especially large ones lying on the periphery underneath the dorsal pleura. They differ,

TABLE I

	Cetacea			Sirenia	
	Delphinus Fiebiger; Lacoste and Baudrimont	*Tursiops* Wislocki	*Phocaena* Lacoste and Baudrimont	*Halicore* Pick	*Trichechus* Wislocki
Pleura	Thick 0.5 mm. (F)	Thick 0.5–1 mm.	Thick	Thin	Thick 0.3–0.5 mm.
Alveoli					
Size	0.8–1 × 0.5 mm. (F)	1–1½ mm.	Not mentioned	Giant alveoli. 6–10 × 1–3 mm.	Peripheral 4 × 6 × 1–2 mm. Central 2–3 × 2–3 mm.
Septa	Heavy	Heavy	Heavy	Heavy	Heavy
Elastic tissue	Moderate	Rich, forming bundles and plexuses	Moderate	Heavy	Relatively little compared to *Tursiops*, condensed on free borders of alveolar septa
Muscle	Little	Little	Little	Heavy bundles	Heavy bundles on free margins of alveolar septa
Capillaries	Double bed	Double bed	Double bed	Not mentioned	Double bed

TABLE I—*Continued*

	Cetacea			Sirenia	
	Delphinus Fiebiger; Lacoste and Baudrimont	*Tursiops* Wislocki	*Phocaena* Lacoste and Baudrimont	*Halicore* Pick	*Trichechus* Wislocki
Bronchioles					
Size.....	No specific details	Slender, long, much branched. Terminal openings 0.1 to 0.2 mm.	No specific details	No specific details	Voluminous, short. Terminal openings 0.3 to 0.6 mm.
Cartilage.....	Into finest bronchioles	Into finest br. Abundant, small, almost continuous links	Into finest bronchioles	Into finest bronchioles	Into finest bronchioles. Fewer, larger, more discontinuous bits than in *Tursiops*
Elastic tissue.	Heavy throughout	Heavy throughout	Heavy throughout	Abundant	Relatively little compared to *Tursiops*
Muscle.....	System of heavy muscle sphincters	System of heavy muscle sphincters	Scant. No system of sphincters	Well developed, but no sphincters	Moderate, but no system of sphincters
Epithelium...	Flat in smallest bronchioles which have respiratory function	Flat in smallest bronchioles which have respiratory function	Cuboidal, without cilia or mucous cells	Flat in smallest bronchioles	Cuboidal or columnar throughout with no respiratory function. Mucous secretion abundant
Blood vessels....	Helicine arterioles (C and B)	No special plexuses	Plexiform veins between groups of alveoli	No mention	No special plexuses

moreover, from the porpoises in not having a system of specialized myo-elastic valves in the smallest bronchioles, as well as by virtue of the fact that the terminal bronchioles are short, wide in diameter, and lined by cuboidal or columnar epithelium.

The lungs of the porpoises themselves, in view of the recent study of *Phocaena* by Lacoste and Baudrimont, are apparently not all equally specialized. Characteristic of them as a group is the possession of a dense pleura and rather large air sacs with stout walls containing much elastic tissue and a double layer of capillaries. The complicated series of muscular valves present in the smallest bronchioles of *Delphinus* and *Tursiops* is not present, however, in *Phocaena*, the small harbour porpoise.

In conclusion the author does not wish to speculate upon the functional implications of the anatomical structures described. This has been done briefly before for the porpoise by Fiebiger (1916) and Wislocki (1929) and for the whales in general in an extensive although entirely speculative manner by Lacoste and Baudrimont (1933). Reference should also be made to the interesting speculations of Irving (1934) on the ability of warm-blooded animals to survive without breathing.

The present study brings out the close similarity in lung structure in the two families of Sirenia. It demonstrates likewise the marked dissimilarities between the sirenian lungs and those of the porpoises belonging to the order of whales. Although there are certain distinctive features common to the lungs of the aquatic mammals thus far investigated, the pronounced differences found to exist indicate that various avenues of specialization have been followed in adapting to aquatic modes of life. The capacity of various aquatic animals to remain submerged and the depths to which they may dive doubtless vary considerably and the degree to which they possess these abilities is probably correlated with differences in lung structure. The Sirenia presumably submerge to lesser depths and are not able to remain under so long as the majority of whales (Parker, 1922, 1932). In this connection it will be of interest to learn eventually how closely the lungs of the large whales resemble those of the porpoises. The seemingly less specialized lungs of the small harbour porpoise (as described by Lacoste and Baudrimont) may be related to the possibility that this small species, in contrast to the larger dolphins (*Delphinus* and *Tursiops*), is not able to submerge for long periods or to attain any great depths. For the present the complicated system of bronchiolar valves possessed only by the two larger species appears to be an ideal specialization to meet the requirements of prolonged submerging and the attainment of considerable depth.

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THE DEVELOPMENTAL STAGES OF LABIDOCERA

MARTIN W. JOHNSON

(From the Scripps Institution of Oceanography of the University of California, La Jolla, California)

INTRODUCTION

Within the past year there has been an increased effort directed towards the study of the development and life histories of marine copepoda. Five publications have recently appeared (Nicholls, 1934; Gurney, 1934; Campbell, 1934; Johnson, 1934*a*, 1934*b*) describing in detail the development of a total of seven marine calanoids. Prior to these works only nine species of this group had been investigated as to their complete development. Gurney (1934) has attributed this lag in investigation of the development of this, the most important of the marine plankton copepod groups, to the difficulty experienced in laboriously tracing out the life histories from the various stages as collected from the plankton, a difficulty further enhanced by the failure of many species to carry eggs. More success has been obtained in the study of fresh water calanoids, due to the greater ease with which they may be cultured in the laboratory. Thus far only one marine calanoid (*Calanus finmarchicus*) has been successfully reared through all the stages to the adult (Lebour, 1916). Gibbons (1933) has cultured this species through the nauplius stages. *Euchaeta norvegica* has been reared through the nauplius stages into the first copepodid; the nauplii apparently being sustained by a large store of yolk (Nicholls, 1934).

The species included in the present study are *Labidocera trispinosa* and *Labidocera jollæ*, which were described by Esterly (1905, 1906) from plankton collections made off the southern California coast. The former is reported as fourth in abundance of the copepods occurring in this region, and is at times very numerous above one hundred fathoms (Esterly, 1912). It is evidently more or less sporadic in occurrence and is found only sparsely in inshore collections. *Labidocera jollæ* is relatively more scarce, judging from the infrequent reference to it in Dr. Esterly's works. My inshore collections indicate that its nauplius stages are as a rule less numerous than those of *Labidocera trispinosa* and that the two species reproduce concurrently during the summer and autumn, and to a lesser extent well into winter. The spring season has not been investigated.

These are the only two species of *Labidocera* reported from this region. The nearest relative whose nauplius larvae might on superficial examination be confused with those of the present species is *Epilabidocera amphitrites*. This species, however, has not been found south of San Francisco Bay and its larval stages are known (Johnson, 1934*b*) and can on careful examination be quite readily distinguished from the present species.

In a preliminary investigation of the seasonal occurrence of zoöplankton at La Jolla, Murphy (1924) concluded that 70–80 per cent of the total plankton was made up of immature forms whose generic identity is not known. Copepod nauplii formed the major part of this percentage, but to what extent the present species of *Labidocera* entered into the computations is not known. Thus far I have found them to play only a minor rôle in local inshore waters.

Procedure

The material used in the present study was obtained from surface plankton collections taken at the Scripps Institution pier. *Labidocera jollæ* female copepodid V was, however, found only in an offshore collection put at my disposal through the courtesy of Dr. E. G. Moberg. This collection also provided material for checking the adult stages of both species. Both living and preserved specimens were employed in working out the successive stages. The identity of the larvæ was established by experimentally rearing the last nauplius stage through the critical moult to the first copepodid stage and linking this with the successive copepodid stages to the adult, which proved to be of the genus *Labidocera*.

The nauplius larvæ of the two local species of Labidocera are separable on small but consistent specific characters mentioned later. A total of six specimens of the last nauplius stage of *Labidocera trispinosa* were reared through the critical moult and the resulting first copepodid examined by complete dissection. Four specimens of *Labidocera jollæ* were thus reared and examined, and many specimens of both species were examined from plankton collections. It is interesting to note that the specific differences appear no greater in the first copepodid stage than in the preceding nauplius stage, but in the following instars the species are easily separated by the clearly visible cephalic hooks present in *Labidocera jollæ* but wanting in *Labidocera trispinosa*, and by the longer first antennae of the latter species.

All drawings were made with the aid of a camera lucida.

LABIDOCERA TRISPINOSA

The Nauplius Stages

The early development is divided into the characteristic six nauplius stages. Pronounced specific differentiation does not appear until the second stage, and each following moult accentuates or brings to light new differentiations.

Contrasted with nearly all copepod nauplii, the larvae of *Labidocera trispinosa* and *Labidocera jollæ* are noticeably elongated. This elongation is, however, only of an intermediate nature when compared with the elongation of the later larvæ of such forms as *Rhincalanus* (Gurney, 1934, Figs. 3, 4, 5) and a local larva tentatively classified as *Pontellopsis occidentalis*. The appendages of *Labidocera trispinosa* are also relatively long and the first antennæ are normally directed straight forward and in contact nearly their whole length. In the later nauplius stages the distal segment of the first antenna is faintly orange in color; the color increasing in intensity toward the tip. Similar pigment is also present in the second antenna, but here the greatest intensity is in the first basipod. The mandibles are nearly colorless. A little of the pigment is visible on the ventral surface of the body and in the alimentary canal where a small splash of red also appears in some specimens. The eye is very dark reddish-brown. The general pigmentation is quite variable in intensity and specimens that have been kept alive in the laboratory a day or longer may become quite colorless.

The labrum is long and rather wide and only very sparsely armed with short weak setæ.

The plumose nature of the appendage setæ can in some instances be seen only with difficulty and is here indicated only in the figures for the sixth nauplius stage.

Nauplius Stage I (Plate I, Fig. 1)

Body.—0.13–0.14 mm. long, oval, posterior end bearing two small spines.

First antenna (Plate IV, Fig. 5).—Three segments, the first short and with one short ventral seta; the second somewhat longer and bearing ventrally one very short and one long seta; the third or distal segment bearing terminally three long setae and ventrally near the tip a short spine.

Second antenna.—First basipod with one small masticatory hook. Second basipod with one small masticatory hook and an adjacent small outwardly directed spine. Endopod of one segment with two

terminal setæ and one lateral seta. Exopod of six segments, the first two fused, the first with no seta, 2–5 with one seta each and the sixth with two setæ.

Mandible.—First basipod with a small rounded chewing process bearing a small spine. Second basipod with two inner spines. Endopod of two completely fused segments indicated only by the presence of three short weak spines on the first and one very short and two long setae on the second. Exopod of four segments, 1–3 with one seta each, the fourth with two setæ.

Nauplius Stage II (Plate I, Fig. 2)

Body.—0.175–0.201 mm. long (average of 11 measurements, 0.185 mm.) terminating posteriorly in one long heavy setose spine and one shorter dorsally directed plumose seta at its right. Partially surrounding the base of each of these is a number of very short fine spines.

First antenna (Plate IV, Fig. 6).—The first and second segments as in I; the distal segment bears at the tip three long plumose setæ and one shorter, lighter accessory seta and the dorsal and ventral margins each bear a separate row of fine hair-like setæ.

Second antenna.—The first basipod with one masticatory hook. Second basipod with one long heavy masticatory hook, an adjacent small outwardly directed spine and one small spine situated distally near the endopod. Endopod with three long terminal setæ and one long lateral seta bearing a small smooth process near its proximal end. Exopod as in I but with two setæ on the second segment.

Mandible.—First and second basipods as in I. Endopod as in I but with increased strength of armature. Exopod as in I but with two setæ on the first segment indicating a segmentation which remains latent until the first copepodid stage is reached.

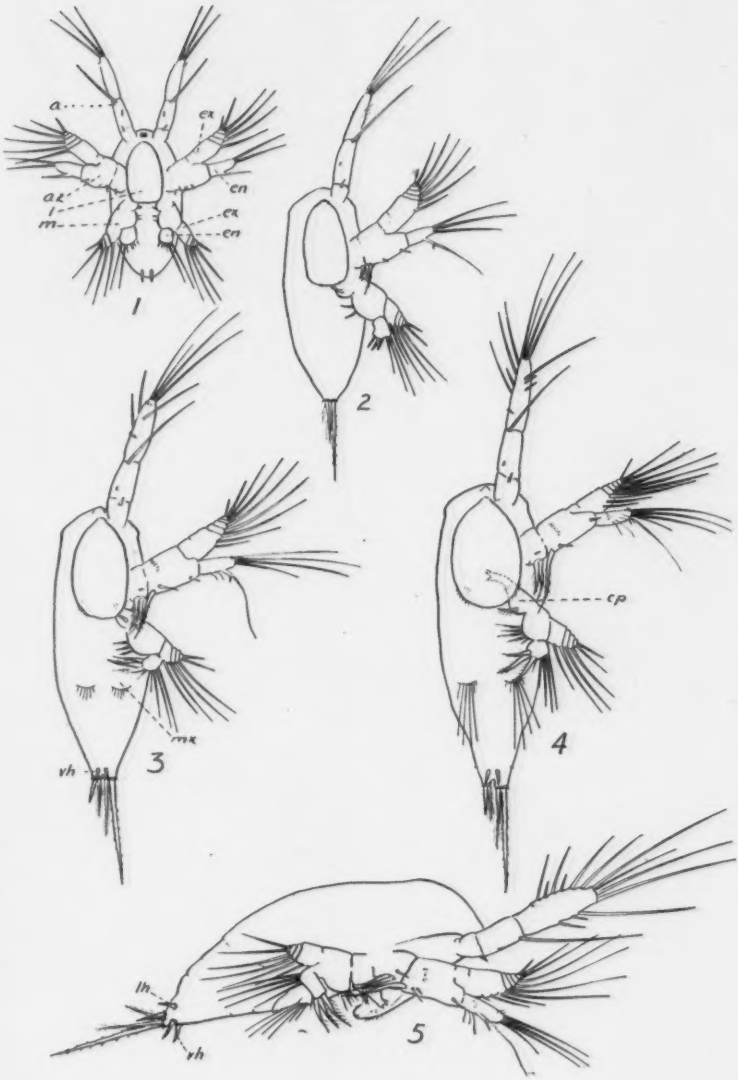
EXPLANATION OF PLATE I

Labidocera trispinosa

FIG. 1. Nauplius Stage I.
FIG. 2. Nauplius Stage II.
FIG. 3. Nauplius Stage III.
FIG. 4. Nauplius Stage IV.
FIG. 5. Nauplius Stage V.

Abbreviations: *a*—first antenna.
a2—second antenna.
cp—chewing process.
en—endopod.
ex—exopod.
l—labrum.
lh—lateral hook.
m—mandible.
mx—first maxilla.
vh—ventral hook.

PLATE I



Nauplius Stage III (*Plate I, Fig. 3*)

Body.—0.215–0.271 mm. in length (average of 23 measurements, 0.235 mm.). Posterior end armed with one long left and one short right terminal setose spine, and one flexible inner plumose seta in conjunction with each spine. Just anterior to the terminal armature there is now one pair of ventral hooks.

First Antenna (Plate IV, Fig. 7).—Segments one and two as in II, the distal segment with four terminal setæ, two long dorsal marginal setæ, one long ventral marginal seta, and a transverse series of minute spines on the inner margin near the middle. The fine hair-like setæ found on the distal segment in II are now wanting.

Second Antenna.—First basipod with two long strong masticatory hooks upon a common base and adjacent to these one small spine. Second basipod as in II but with also a transverse series of minute spines situated on the ventral proximal portion of the segment. Endopod as in II but with four terminal setæ and two additional fine hair-like setæ near the origin of the single long lateral seta. Exopod as in II but with three setæ each on the second and terminal segments.

Mandible.—First basipod as in II. Second basipod with three inner spines. Endopod with three strong hook-like spines and one very weak basal seta on the first segment and four slender setæ on the second segment. Exopod as in II.

First Maxilla.—Bud fringed with short hairs, discernible only with difficulty in some specimens.

Nauplius Stage IV (*Plate I, Fig. 4*)

Body.—0.250–0.325 mm. long (average of 14 measurements 0.278 mm.). The caudal armature is like that in Stage III with the addition of a series of minute lateral spines marking the location where the lateral hooks are destined to appear in the following stage.

First Antenna (Plate IV, Fig. 8).—First and second segments unchanged; distal segment with four terminal setæ, one short and three long dorsal marginal setæ, one long and two short ventral marginal setæ and a transverse series of minute spines on the inner side.

Second Antenna.—First and second basipods as in III. Endopod as in III but with also a few very minute spines grouped on the lateral surface. Exopod as in III.

Mandible.—First basipod with the chewing process much enlarged and terminating in two teeth and a small lateral seta. Proximally the process bears a heavy setose spine. Second basipod with five inner spines. Endopod and exopod as in III.

First Maxilla.—Bud with slender weakly chitinized setæ.

Second Maxilla.—Poorly defined bud. (Visible only in some specimens nearing ecdysis.)

Nauplius Stage V (Plate I, Fig. 5)

Body.—0.310–0.370 mm. in length (average of 14 measurements, 0.332 mm.). Posterior end armed as in IV but including also one pair of lateral hooks similar to the ventral pair.

First Antenna (Plate IV, Fig. 9).—Unchanged except for increase to four long and two short (alternating with the long) dorsal marginal setæ and one long and two short ventral marginal setæ.

Second Antenna.—First basipod unchanged. Second basipod unchanged but for addition of one very small spine adjacent to the masticatory hook, thus making two small spines in this location. Endopod as in IV but with three fine hair-like setæ near the base of the single long lateral seta. Exopod as in IV but with four setæ on the second segment.

Mandible.—As in IV.

First and Second Maxilla.—Rudimentary.

Maxilliped.—Bud.

Nauplius Stage VI (Plate II, Fig. 1)

Body.—0.360–0.420 mm. long (average of 11 measurements, 0.386 mm.). Caudal armature as in Stage V. Just anterior to the lateral hooks there is on each side a series of very minute lateral spines. The rudimentary legs which in some specimens of Stage V can be seen as blocks of undifferentiated tissue are now quite distinct, but the first maxillæ are still poorly defined.

First Antenna (Plate IV, Fig. 10).—As in Stage V but with a total of five ventral marginal setæ on the distal segment.

Second Antenna.—First and second basipods as in V. Endopod as in V but with a total of five terminal setæ. Exopod as in V.

Mandible.—As in Stage V but with increased strengthening of the masticatory portions and with a total of six setæ on the inner margin of the second basipod.

First and Second Maxilla.—Rudimentary.

Maxilliped.—Bud.

First and Second Legs.—Rudimentary.

The Copepodid Stages

Following the critical moult there is a succession of six copepodid stages, the last of which is the adult animal. Living animals display a

rather faint dark orange coloration of the first and second basipods of the second antennæ, and some of this pigment is also evident in the posterior end of the body. A few dark spots occur along the mid body and a faint green is noted along the alimentary canal. The color intensity varies greatly and is usually more pronounced in the older specimens.

From the first copepodid stage, the animals swim in the fashion apparently typical of the *Pontellidæ*, i.e. with quick sweeps of the second antennæ and with rhythmic dorsal ventral motion of the urosome. Thus, when swimming leisurely, the animal gives the appearance of a hovering bird.

Copepodid Stage I (Plate II. Fig. 2)

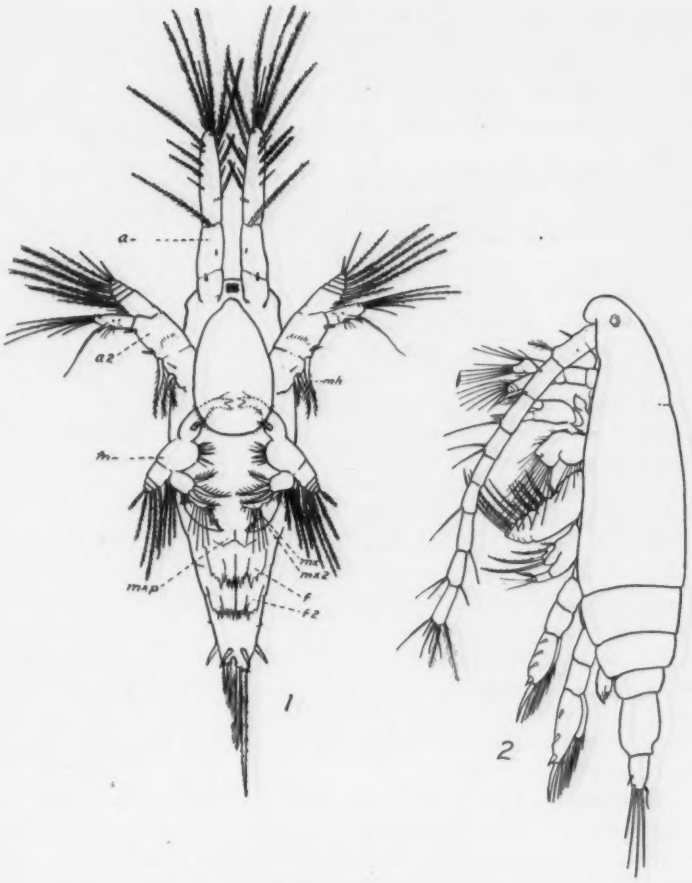
Length 0.498–0.590 mm. (average of 5 measurements 0.543 mm.) to the end of caudal rami. Thorax [1] of four segments, abdomen of one segment. The abdomen and caudal rami are symmetrical and each ramus bears four long terminal setæ, one short lateral seta (which in the later stages assumes the character of the regular terminal setæ), and one dorsal seta. Dorsal cuticular lenses are poorly defined and partially surrounded by pigment bodies. The rostral processes which are typical of the later stages are wanting, the rostrum being only a rounded blunt protuberance.

First Antenna.—Nine evident segments.

Second Antenna.—First basipod with one seta. Second basipod with two setæ. Endopod of two segments, the first fused with the second basipod, and bearing two outer setæ, the second segment forming a lateral and a terminal lobe each bearing a distinct group of setæ indicating the fusion of two segments homologous to the two clearly defined end segments of the endopod of the mandible. The lateral lobe bears a group of three setæ, and the terminal lobe, six setæ. The exopod consists of five evident segments, the first short and bearing one inner seta, the second long and bearing three setæ. The remaining end segments are short and very obscure and bear a total of eight setæ.

[1] The term 'thorax' used in connection with these species designates that portion of the body bearing visible feet plus the next segment to the posterior. Thus the adult animal is considered to possess a total of six thoracic segments of which the genital segment is the last to appear.

PLATE II



Labidocera trispinosa

FIG. 1. Nauplius Stage VI.
FIG. 2. Copepodid Stage I, lateral.

Abbreviations: *a*—first antenna.
a2—second antenna.
f—first legs.
f2—second legs.
m—mandible.
mh—masticatory hook of second basipod.
mx—first maxilla.
mx2—second maxilla.
mxp—maxilliped.

Mandible.—Mandibular blade with four teeth, a small spine, and several fine short setæ. Palp with a very short first basipod; a long second basipod with four slender setæ; an endopod of two short segments, the first with a distal group of four long setæ, and the second with a terminal group of six long setæ; an exopod of five segments, 1–4 with one long seta each and the fifth with two shorter slender setæ.

First Maxilla.—The structure of this appendage is like that of the adult except for less obvious segmentation and smaller number of setæ on certain lobes. Gnathobase or first inner lobe with eleven short spines; second inner lobe with three long strong setæ; epipod or outer setiferous plate bears four long slender setæ; the third inner lobe situated on a very short segment bears three long setæ, while the outer margin of this segment bears a single long seta. The following segment bears three slender inner setæ and is fused with the first segment of the exopod which bears three lateral setæ. The second or terminal segment of the exopod bears five long coarse setæ. Endopod of two poorly defined segments, the terminal one with seven long coarse setæ.

This appendage is identical with that of *Labidocera jollæ* which is figured for the adult in Plate IV, Fig. 13.

Second Maxilla.—Uniramose and strongly built. First basipod with two endites each bearing one short and one long seta and a number of fine spines at their bases. Second basipod with two endites each with one short and two long setæ; the first endite also bears a number of very fine spines. Endopod of five poorly defined segments, the first segment slightly the longest and bearing one long and one short seta, segments 2–4 each with one long seta, and the fifth segment with one long and one short seta. All the setæ of the appendage are strong and coarsely setose.

Maxilliped.—Uniramose. The first basipod enlarged and consisting of three lobes, the first with one short setose seta, the second with one long and one short setose seta, the third with one long setose seta. The second basipod is narrow and bears no setæ. Endopod of two segments, the first with one distal plumose seta, the second with three terminal smooth setæ.

First Leg.—First and second basipod with no setæ. Endopod of one segment with seven setæ. Exopod of one segment with four outer spines, a terminal blade and three setæ.

Second Leg.—First and second basipods with no setæ. Endopod of one segment with six setæ. Exopod of one segment with three outer spines, a terminal blade and three setæ.

Third Leg.—Rudimentary.

Copepodid Stage II

Length 0.720–0.797 mm. (average of 5 measurements, 0.767 mm.). Thorax of five segments, abdomen of one segment. Dorsal cuticular lenses and rostral processes present. Urosome and caudal rami symmetrical.

First Antenna.—Fourteen segments.

Second Antenna.—As in I but with five setæ on the lateral lobe and six setæ on the terminal lobe of the distal segment of the endopod.

Mandible.—As in I but with five mandibular teeth, and with six setæ on the terminal segment of the endopod.

First Maxilla.—As in I but with six setæ on the epipod and four setæ on the third inner lobe.

Second Maxilla.—As in I.

Maxilliped.—As in I but with two short setæ on the first lobe of the first basipod; and with an endopod of three segments, the first with one long and one short plumose seta, the second with one long plumose seta, and the third with three long smooth setæ.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with three outer spines, a terminal blade, and four setæ.

Second Leg.— First basipod with one seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with two outer spines, a terminal blade, and four setæ.

Third Leg.—First and second basipods with no setæ. Endopod of one segment with six setæ. Exopod of one segment with three outer spines, a terminal blade, and three setæ.

Fourth Leg.—Rudimentary.

Copepodid Stage III

Length 1.03–1.11 mm. (average of 5 measurements 1.064 mm.). Thorax of six segments, abdomen of one segment. Posterior end of body symmetrical.

First Antenna.—Nineteen segments.

Second Antenna.—As in II.

Mandible.—As in II.

First Maxilla.—As in II but with eight setæ on the epipod and four setæ on the first segment of the endopod.

Second Maxilla.—As in II.

Maxilliped.—As in II but with two short plumose setæ at the base of the one long seta on the third lobe of the first basipod, and with an endopod of five segments. The first segment of the endopod is very short and indistinctly defined and bears two long plumose setæ; the second segment is long and bears one plumose seta; the third and fourth segments are shorter and each bear distally one plumose seta; the fifth segment is very short and terminates in three smooth setæ.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade and four setæ.

Second Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade and five setæ.

Third Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with two outer spines, a terminal blade, and four setæ.

Fourth Leg.—First and second basipods with no setæ. Endopod of one segment with six setæ. Exopod of one segment with three outer spines, a terminal blade and three setæ.

Fifth Leg.—Rudimentary.

Copepodid Stage IV

Length female 1.34–1.49 mm. (average of 8 measurements, 1.40 mm.); male 1.23–1.41 mm. (two specimens). Thorax of six segments, abdomen of two segments. The corners of the fifth thoracic segment are symmetrical and slightly pointed, and the urosome is also symmetrical.

First Antenna.—Twenty-three segments.

Second Antenna.—As in III but with seven setæ on the lateral lobe of the terminal segment of the endopod, and with an additional slender seta on the terminal segment of the exopod.

Mandible.—As in III but with seven setæ on the terminal segment of the endopod.

First Maxilla.—As in III but the epipod and exopod each with nine setæ.

Second Maxilla.—As in III but with the addition of two setæ at the base of the two long setæ on the first endite of the first basipod.

Maxilliped.—As in III but with an additional small seta at the base of the two longer setæ on the second lobe of the first basipod. The second basipod is finely serrate on the anterior surface, and there are

now two plumose setæ on the distal end of the second segment of the endopod.

This appendage is now essentially the same as in the adult stage (Plate IV, Fig. 23). The short first segment of the endopod, however, becomes so obscure by fusion to the second segment in the later stages that the endopod appears to have only four segments.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade, and four setæ.

Second Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with ten setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade, and five setæ.

Third Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade, and five setæ.

Fourth Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with three outer spines, a terminal blade, and five setæ.

Fifth Legs, Female (Plate IV, Fig. 14).—Biramose, small and symmetrical. The second basipod bears on its posterior surface a short plumose seta. Endopod represented by a short smooth segment. Exopod of one segment with one outer spine near the middle and one near the tip. At the tip there is one terminal spine and adjacent to it on the inner side is a very small spine which in the later stages develops into the larger terminal point.

Fifth Legs, Male (Plate IV, Fig. 19*a*).—Biramose and asymmetrical. The second basipod and the endopod are like the corresponding parts in the female. Right exopod slightly longer than the left and with two segments indicated. Each exopod bears two small outer spines and a terminal point.

In this stage the sexes apparently can be distinguished only by the structure of the fifth legs.

Copepodid Stage V

Length, female 2.04–2.06 mm. (three measurements). Thorax of six segments, abdomen of two segments. The fifth thoracic segment with sharply pointed corners. Posterior portion of body symmetrical and without protuberance on genital segment. No males were found of this species in this stage.

First Antenna.—Twenty-three segments discernible.

Second Antenna.—As in IV.

Mandible.—As in IV with setæ of terminal endopod segment increased to eight.

First Maxilla.—Segmentation more defined and increased strength of armature.

Second Maxilla. As before but with a total of five setæ on the first endite of the first basipod.

Maxilliped.—No change in structure.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with six setæ. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with two outer spines, a terminal blade, and four setæ.

Second Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with eight seta. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with three outer spines, a terminal blade, and five setæ.

Third Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with eight setæ. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with three outer spines, a terminal blade, and five setæ.

Fourth Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with seven setæ. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with three outer spines, a terminal blade, and five setæ.

Fifth Leg, Female (Plate IV, Fig. 15).—Similar to Stage IV, with the small inner terminal spine enlarged to a heavy terminal point.

Copepodid Stage VI, Adult

The original description given for the adult stage of this and the following species is not complete. It is therefore desirable to include here also brief descriptions of this stage.

Female (Plate V, Fig. 1).—Length 2.50–2.82 mm. (average of 7 measurements, 2.81 mm.). The measurements given by Esterly (1905, p. 202) for the adult female is 1.6 mm.; only a little greater than is here given for copepodid Stage IV. The structure of the adult appendages and body segmentation is essentially the same as in Stage

V. In Plate IV are figured the fifth legs (Fig. 16), the mandible (Fig. 12), and the maxilliped (Fig. 23). The last two named appendages are the same in both sexes.

Male (Plate V, Fig. 2).—Length 2.14–2.47 mm. (average of 7 measurements, 2.31 mm.). This agrees rather closely with Esterly's maximum figure which is 2.2 mm. The abdomen consists of four segments. The right posterior corner of the fifth thoracic segment is produced into a slender curved spine, and adjacent to it on the posterior margin on the same side there are two to three smaller spines. Esterly (1905, p. 202) notes only two such spines. The fifth legs (Plate IV, Fig. 22) are as in the original description but with a rudimentary endopod on the left foot.

Labidocera jollæ

The adults of *Labidocera jollæ* and *Labidocera trispinosa* are easily distinguished by sharply defined characters, but the nauplius larvæ of the species are very similar and can be distinguished only by small but yet definite differences.

Each developmental stage of *Labidocera jollæ* was also worked out carefully and will be briefly compared with those of *Labidocera trispinosa* in all the essential characters, stressing mainly the points of difference.

Nauplius Stage I was not found or could not be distinguished from the corresponding stage referred to *L. trispinosa*. (The nauplius examined was referred to this species in view of its greater numbers.) A study of the following nauplius stages will show that the separation of the two species in Stage I must depend mainly, if not wholly on the comparative slenderness of the masticatory hooks occurring on the first and second basipod of the second antenna. These hooks are only weakly developed in this stage.

In the following nauplius stages *L. jollæ* is usually slightly the larger of the two species, the first antennæ and the long caudal spine are relatively shorter, and the pigmentation of the body is more pronounced and of a definite blue-green cast. When at rest the first antennæ are directed straight forward.

Nauplius Stage II (Plate III, Fig. 1)

Length 0.200–0.211 mm. (two specimens measured). The anatomical feature distinguishing the species in this stage is a very slender masticatory hook on the second basipod of the second antenna as compared with the heavy, well developed corresponding hook in *L. trispinosa* (Plate IV, Figs. 1 and 2, *mh*). That this character is a

constant one is supported by its persistence in combination with other specific characters that will be mentioned for the later stages. The short spine appearing on the distal portion of the second basipod of the second antenna of *L. trispinosa* is wanting in *L. jollæ*. Its first appearance is in Stage III. This spine and also the other spines occurring on the second antenna are more slender in the latter species in all stages.

Nauplius Stage III

Length 0.260–0.270 mm. (three measurements). The only additional difference in this stage is the appearance of a transverse series of very minute spines on the second basipod of the second antenna. In *L. jollæ* the series is located on the distal half of the segment while in *L. trispinosa* it is on the proximal half. This difference persists throughout all of the stages but in the older larvæ a few similar small spines appear also on the proximal half of the segment in *L. jollæ*.

Nauplius Stage IV

Length 0.265–0.319 mm. (average of six measurements 0.304 mm.). In this stage the development of the larval chewing process is completed in both species. In *L. jollæ* the process terminates in three teeth and a small spine, while in *L. trispinosa* it terminates in only two teeth and a small spine (Plate I, Fig. 4, *cp;* Plate IV, Figs. 3 and 4). The greater number of mandibular teeth in the former species is reflected in the copepodid stages, in the first of which the number is in the same ratio as in the nauplius stage.

Nauplius Stage V

Length 0.390–0.426 mm. (average of five measurements 0.398 mm.). No added specific changes.

Nauplius Stage VI (Plate III, Fig. 2)

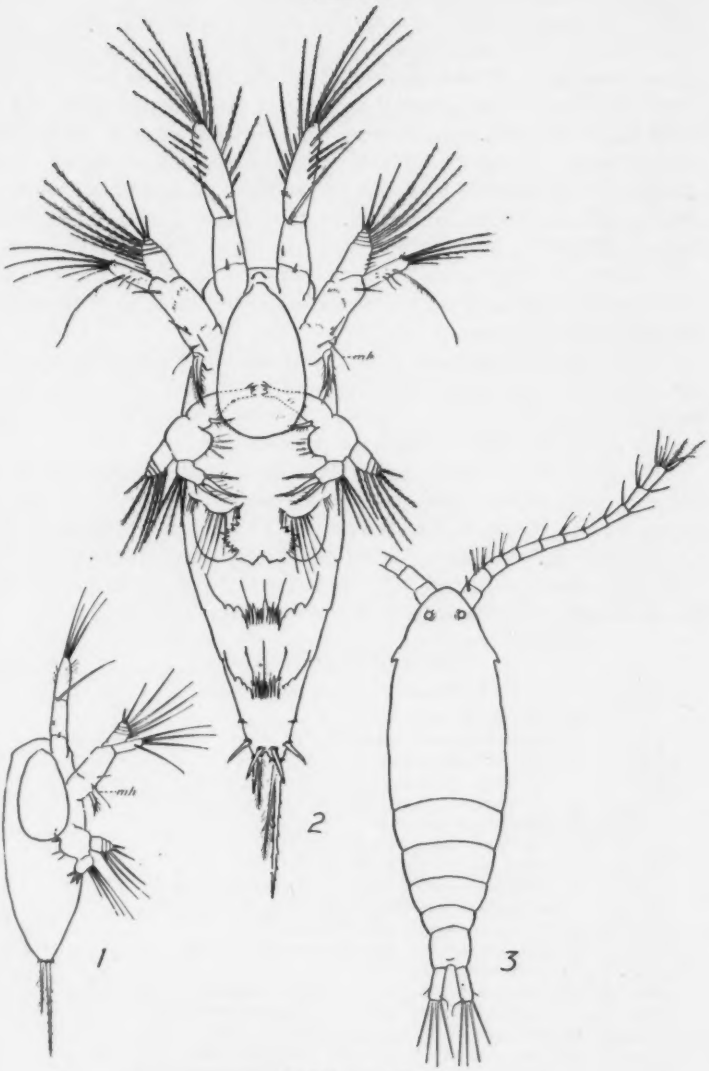
Length 0.424–0.495 mm. (average of eleven measurements, 0.472 mm.).

In this stage there appears an additional short dorsal marginal seta on the distal segment of the first antenna, bringing the total number of dorsal marginal setæ in *L. jollæ* to seven, whereas in *L. trispinosa* the total number in this stage is six, the same as in the fifth nauplius stage.

The Copepodid Stages

In the first copepodid stage of *Labidocera jollæ*, the cephalic hooks and also the rostral processes are wanting. Hence in this stage it is

PLATE III



Labidocera jollæ

FIG. 1. Nauplius Stage II.
FIG. 2. Nauplius Stage VI.
FIG. 3. Copepodid Stage II, dorsal.
Abbreviation: *mh*—masticatory hook of second basipod.

practically indistinguishable from the same stage in *L. trispinosa* except for small differences occurring in the mandibular blade and in the endopod of the maxilliped. In the former species the mandibular blade bears six teeth as compared with only four in the latter species, and in the former the terminal segment of the endopod of the maxilliped bears but two smooth setæ, while in the latter it bears three smooth setæ. These distinctions remain evident through all of the stages, but in Stage II each species acquires an additional tooth on the mandibular blade, giving the total number found in the adult animal (Plate IV, Figs. 11 and 12). In all of the copepodid stages *L. jollæ* is relatively more green in color. The development of the appendages and the body segmentation is the same in both species for corresponding stages.

In the second copepodid stage (Plate III, Fig. 3), *L. jollæ* acquires the rostral processes and also the cephalic hooks. The presence of cephalic hooks serves as a reliable and easy distinction between the species in this and all the following stages.

The sexes are first distinguishable in the fourth copepodid stage by the structure of the fifth legs. The development of these legs is shown for the female copepodid IV and VI in Plate IV, Figs. 17 and 18 respectively. In Stage V, not figured, the legs are almost identical with those shown for Stage VI. In Plate IV, Figs. 19 to 21, is given the development of the male fifth legs from Stage IV to VI.

EXPLANATION OF PLATE IV

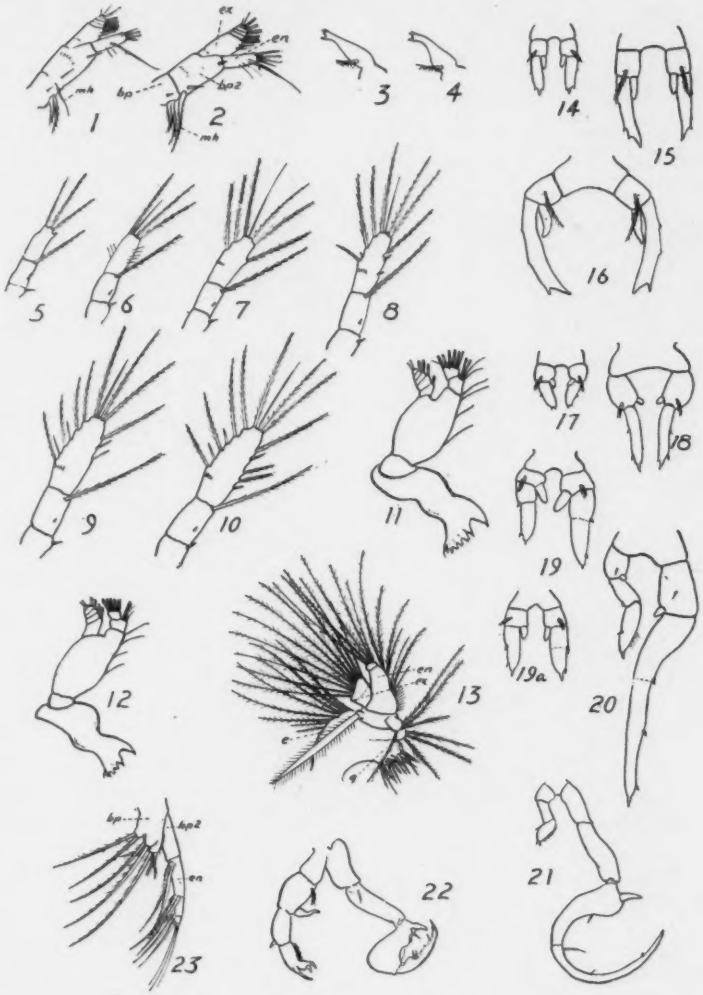
Labidocera trispinosa and *L. jollæ*.

FIG. 1. *L. jollæ*, second antenna.
FIG. 2. *L. trispinosa*, second antenna.
FIG. 3. *L. jollæ*, chewing process.
FIG. 4. *L. trispinosa*, chewing process.
FIGS. 5–10. *L. trispinosa*, first antenna nauplius Stages I–VI.
FIG. 11. *L. jollæ*, adult mandible.
FIG. 12. *L. trispinosa*, adult mandible.
FIG. 13. *L. jollæ*, adult first maxilla.
FIG. 14. *L. trispinosa*, female fifth legs, copepodid Stage IV.
FIG. 15. *L. trispinosa*, female fifth legs copepodid Stage V.
FIG. 16. *L. trispinosa*, female adult fifth legs.
FIG. 17. *L. jollæ*, female fifth legs, copepodid Stage IV.
FIG. 18. *L. jollæ*, female adult fifth legs.
FIG. 19. *L. jollæ*, male fifth legs, copepodid Stage IV.
FIG. 19a. *L. trispinosa*, male fifth legs, copepodid Stage IV.
FIG. 20. *L. jollæ*, male fifth legs, copepodid Stage V.
FIG. 21. *L. jollæ*, male adult fifth legs, (reduced scale).
FIG. 22. *L. trispinosa*, male adult fifth legs, (reduced scale).
FIG. 23. *L. trispinosa*, adult maxilliped.

Abbreviations: *bp*—first basipod; *bp2*—second basipod; *e*—epipod; *en*—endopod; *ex*—exopod; *g*—gnathobase; *mh*—masticatory hook of second basipod

A comparison of body sizes shows that during the nauplius and early copepodid stages, *L. jollæ* is slightly larger than *L. trispinosa*. In copepodid Stages IV and V the measurements are about equal, but in the adult stage the latter is a little longer.

PLATE IV



The adult of *L. jollæ* agrees essentially with the original description given by Esterly (1906, p. 74).

Female.—Length 2.49–2.67 mm. (average of nine measurements, 2.57 mm.). In this stage the urosome is very asymmetrical (Plate V, Fig. 8), an asymmetry which is first indicated only in the broadening of the right caudal ramus of Stage V (Plate V, Fig. 7).

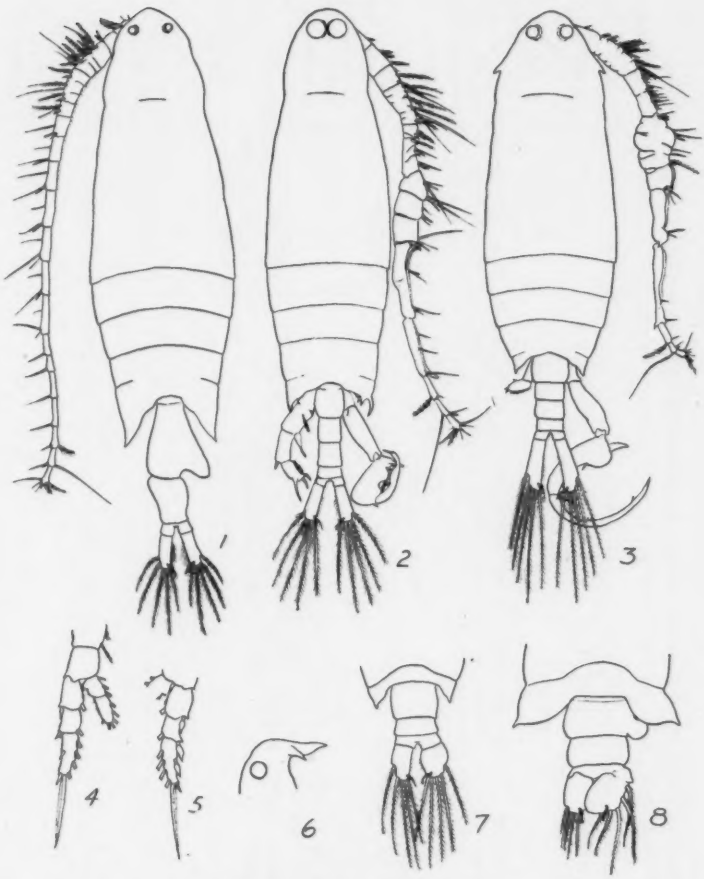
Male.—Length 2.06–2.39 mm. (average of twenty-eight measurements, 2.20 mm.). The rostrum is asymmetrical due to the right prong being much reduced (Plate V, Fig. 6). Esterly also noted this asymmetry but was not certain if it should be considered an individual deformity or a distinct character, since his description was based on a single specimen. I have examined many adult males and find this peculiar asymmetry to be constant in every specimen examined. In the fifth copepodid stage the male rostrum is, however, symmetrical. Another unusual asymmetry found only in the adult male is evident in the modification of the outer spines occurring on the exopod of the right first leg (Plate V, Fig. 5). These are broader and more leaf-like than the regular outer spines found on the other swimming legs (Plate V, Fig. 4).

Remarks

It has been shown by different workers that the nauplius larvæ of marine copepods belonging to the same genus are, as one might expect, strikingly similar, and may in some instances be identical, as is shown by Oberg (1906) and Gurney (1931) for *Acartia longiremis*, *A. bifilosa*, and *A. clausi*.

In view of the great likeness found in the larvæ of related copepods, the two larvæ herein described are doubtless typical of the genus *Labidocera* and, perhaps with some modifications, of the whole family *Pontellidæ*. Thus far, however, we have information on only three genera of the group, namely, *Labidocera*, *Epilabidocera* (syn. *Paralabidocera*), and *Pontella* (incomplete). The larvæ of the present species of *Labidocera* are very similar to the corresponding stages of *Epilabidocera amphitrites* recently described (Johnson, 1934*b*), and judging from the few figures given by Claus (1893) for *Pontella mediterranea* there appears to be here also essential agreement as to type. An elongated body is common to the three genera, though much accentuated in *Pontella*. The arrangement and type of caudal armature is the same in the investigated species of *Labidocera* and *Epilabodocera*, and agrees with *P. mediterranea* but for the exaggerated size of the left terminal spine in the latter. The first antennæ of the three genera agree as to segmentation and shape and the armature of

PLATE V



Labidocera trispinosa and *L. jollæ*

FIG. 1. *L. trispinosa*, adult female, dorsal.
FIG. 2. *L. trispinosa*, adult male, dorsal.
FIG. 3. *L. jollæ*, adult male, dorsal.
FIG. 4. *L. jollæ*, adult male left first leg.
FIG. 5. *L. jollæ*, adult male exopod right first leg.
FIG. 6. *L. jollæ*, adult male head, lateral.
FIG. 7. *L. jollæ*, female copepodid V posterior end, dorsal.
FIG. 8. *L. jollæ*, adult female posterior end, dorsal.

TABLE I

Identification Table for Nauplius Stages of Labidocera trispinosa and L. jollæ

Stage		I	II	III	IV	V	VI
Length in mm.	*L. trispinosa*	0.130–0.140	0.175–0.201	0.215–0.271	0.250–0.325	0.310–0.370	0.360–0.420
	L. jollae	—	0.200–0.211	0.260–0.270	0.265–0.319	0.390–0.426	0.424–0.495
First Antenna distal segment	*L. trispinosa*	3 terminal setae	4 terminal setae, 1 dorsal and 1 ventral series of fine hairs	4 terminal setae, 2 dorsal and 1 ventral marginal setae (fine hairs wanting)	As in III, but 4 dorsal and 3 ventral marginal setae	As in IV, but 6 dorsal and 3 ventral marginal setae	As in V, but 6 dorsal and 5 ventral marginal setae
	L. jollae	—	As above	As above	As above	As above	As above, but 7 dorsal and 5 ventral marginal setae
Caudal Armature		2 small end spines	1 long end spine and 1 shorter end seta	1 long and 1 short end spine, 2 end setae, and 1 pair ventral hooks	As in III	As in IV, but also 1 pair lateral hooks	As in V
First Maxilla		0	0	Bud	Bud	Rudimentary	Rudimentary
Second Maxilla		0	0	0	Bud	Bud	Rudimentary
Maxilliped		0	0	0	0	Bud	Bud
First and second legs		0	0	0	0	0	Rudimentary

TABLE II

Identification Table for Copepodid Stages of Labidocera trispinosa and L. jollæ

Stage		I	II	III	IV	V	VI
Length in mm.	*L. trispinosa*	0.498–0.590	0.720–0.797	1.03–1.11	1.34–1.49 ♀ 1.23–1.41 ♂	2.04–2.06 ♀	2.50–2.82 ♀ 2.14–2.47 ♂
	L. jollae	0.570–0.650	0.855–0.990	1.20–1.215	1.34–1.50 ♀ 1.35–1.43 ♂	1.97–2.00 ♀ 1.71–1.78 ♂	2.49–2.67 ♀ 2.06–2.39 ♂
Head	*L. trispinosa*	Rostral processes and cephalic hooks wanting	Rostral processes present, cephalic hooks wanting	As in II	As in III	As in IV	As in V
	L. jollae	Rostral processes and cephalic hooks wanting	Rostral processes and cephalic hooks present	As in II	As in III	As in IV	As in V
Thoracic Segments		4	5	6	6	6	6
Abdominal Segments		1	1	1	2	2 ♀ 3 ♂	2 ♀ 4 ♂
Mandibular teeth	*L. trispinosa*	4	5	5	5	5	5
	L. jollae	6	7	7	7	7	7
Legs present		1st and 2nd (3rd rudimentary)	1st, 2nd, and 3rd (4th rudimentary)	1st, 2nd, 3rd and 4th (5th rudimentary)	1st, 2nd, 3rd, 4th and 5th (modified)	As in IV	As in V

at least the species of *Labidocera* and *Epilabidocera* is essentially the same. The number of ventral marginal setæ is the same in all three species, but *E. amphitrites* possesses a total of eight dorsal marginal setæ, as opposed to seven in *L. jollæ* and six in *L. trispinosa*. An alternation of long and short setæ in the dorsal marginal series is common to all three species. It is also characteristic of the living larvæ of all three species to hold the first antennæ extended straight forward and in contact when not swimming.

In Tables I and II are given the characters most useful in identification of the various nauplius and copepodid stages.

Summary

1. The developmental stages of *Labidocera trispinosa* and *L. jollæ* are described and figures given.

2. Each species passes through the typical six nauplius and six copepodid stages.

3. It is probable that in the first nauplius stage the species are indistinguishable.

4. In the adult condition the species are sharply distinguished, but during the second to sixth nauplius stages and during the first copepodid stage they are separable only by small but yet definite specific characters, the most useful of these being the type of masticatory hook occurring on the second basipod of the second antenna.

5. Specific identification of the nauplius larvæ was established by experimentally rearing the sixth nauplius stage through metamorphosis.

6. Tables I and II are given to facilitate identification of the nauplius and copepodid stages.

7. The nauplius larvæ of *Labidocera* are very similar to the larvæ of *Epilabidocera* and are believed to typify the nauplius larvæ of at least other nearly related genera of Pontellidæ.

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THE MORPHOLOGY OF GONYOSTOMUM SEMEN FROM WOODS HOLE, MASSACHUSETTS

FRANCIS DROUET AND AARON COHEN

(From the Departments of Botany, Marine Biological Laboratory, University of Missouri, and Harvard University)

The rarely observed flagellate, *Gonyostomum Semen* (Ehrenb.) Diesing,[1] was collected during July, 1934 in a plankton haul from a sphagnum swamp near Woods Hole, Massachusetts, by Miss Hannah T. Croasdale. The swamp is one of a number of sphagnum bogs found on Cape Cod and the nearby islands. Peculiar to it is a dense growth of the white cedar, *Chamæcyparis thyoides* (L.) BSP., various species of Ericaceæ, etc., which extend throughout the broad shallow margin to the edge of a pond in the center. The locality is commonly referred to by residents of Woods Hole as the Cedar Swamp. It is situated about a quarter of a mile east of the village on Nobska Road.

The waters of the pond appear brownish even in thin layers in transmitted light and are almost black in reflected light. Miss Croasdale, who made monthly pH determinations during the past four summers (1931–1934) in open water in various parts of the swamp, says that the pH remains consistently between 4.4 and 4.6. Our own determinations made during the summer of 1934 lie within this range. The bottom of the pond is covered with decomposing vegetable debris in a finely divided state, so that even when slightly agitated the water becomes opaque. The southern part of the pond, where our collections were taken, is shaded for most of the day by the dense growth of white cedar, etc.; little direct sunlight reaches this part of the lake except for a few hours at midday. Collections taken early in the morning (seven o'clock Standard Time) invariably contained so many individuals of *Gonyostomum* that the brownish water became green in the net and collecting bottle. Collections taken at noon or thereabout

[1] GONYOSTOMUM SEMEN (Ehrenb.) Diesing, *Sitz.-ber. k. Akad. Wiss. zu Wien*, **52**: 332. 1865.

Monas Semen Ehrenberg, *Ber. Verh. k. preuss. Akad. Wiss. in Berlin*, **1853**: 191. 1853.

Rhaphidomonas Semen (Ehrenb.) Stein, Organismus der Infusionsthiere 3 (1). Taf. XIII, Fig. 6–12. 1878.

Since botanical nomenclature among the flagellates begins with Linnaeus' *Species Plantarum*, 1753, according to the decisions reached by the Third International Botanical Congress (1910), we must accept the earliest valid name, *Gonyostomum* Diesing, if we are to keep the group separate from the genus *Monas* Ehrenb.

brought few individuals of *Gonyostomum*, but many rotifers and trachelomonads. These observations led us to suppose that the organisms are heliophobic and that a diurnal migration takes place to and from the deep waters according to the intensity of the sunlight which strikes the surface. Lemmermann (1910), on the other hand, describes *Gonyostomum Semen* as 'photophilic.' Further observations are necessary to clear up this matter.

Collections were taken by pouring approximately five gallons of the swamp water, uncontaminated with decomposed material from the bottom, through a net of No. 20 silk bolting-cloth and straining until 200–300 cc. of the liquid remained. This quantity was poured into wide-mouthed, unstoppered bottles and taken at once to the laboratory. When placed in diffused light, the organisms lived in an apparently healthy condition for two or three weeks. By the end of this period, either the rotifers had consumed all of the individuals of the culture or other changes in the medium became so pronounced that the remaining *Gonyostomum* cells burst or encysted. In these cultures in the laboratory, division and, later, encystment of the vegetative cells were observed.

The literature is chiefly vague and non-committal concerning the details of morphology and physiology of *Gonyostomum Semen*, as likewise of other members of the Chloromonadophyceæ (Chloromonadida) with the possible exception of *Vacuolaria virescens* Cienk. (Cienkowsky, 1870; Senn, 1900; Bütschli, 1883). Perhaps this lack of information is due to the apparent rarity of the organisms, which have been reported infrequently since their first recognition. The actual localities for which we find our species previously recorded are three: a sphagnum bog near Berlin (Ehrenberg, 1853; Stein, 1878), a bog near Fölisö in Finland (Levander, 1894), and 'Sphagneten' near Seefeld and Larss in the Tyrol (Dalla Torre and Sarnthein, 1901). Pascher (1913) says of its distribution: "In stehenden Gewässern, Tümpeln und Torfsümpfen; verbreitet, doch vereinzelt." Even if others have observed the organism at other stations, Ehrenberg, Stein, and Levander appear to be the only workers who have contributed to our previous knowledge of the species.

These authors, and compilers of works on the flagellates after them, disagree on certain important details of morphology and physiology of *Gonyostomum Semen*. The flagella, for example, are described variously. Ehrenberg (p. 191) says ". . . ciliis pluribus vibrante." Diesing (1865), who obviously did not see the organism himself, remarks of the genus, "flagello ignoto." Stein mentions and figures two flagella, one projected forward and one trailing.

Levander figures only the forward-projecting flagellum and does not commit himself as to the presence of a trailing one. Similar uncertainty exists concerning the long slime-threads produced from trichocyst-like rods embedded in the cytoplasm. Lemmermann and Pascher place upon Levander all responsibility for the idea that the slime-threads become several times the length of the body of the organism. We find the following statement in Ehrenberg's original diagnosis: "Facile diffluendo ovula glandulam et spiculas bacillares tenues ostendit." It is probable, as Kent (1880) has pointed out, that Ehrenberg's phrase "ciliis pluribus" quoted above refers to the slime-threads when only partially discharged. Likewise, the literature is vague or contradictory as to the function of the triangular cavity and the nature and points of discharge of the contractile vacuoles. Cell division and encystment have not heretofore been described.

Morphology of the Vegetative Cell

In shape and size, the vegetative cells of *Gonyostomum Semen* actually vary more widely than other authors intimate. Generally speaking, the cell body (Figs. 1 and 2) is ovoid and somewhat flattened dorsoventrally, with a short, cylindrical, more or less pointed caudus at the posterior end. The dorsal surface may be ovate, obovate, ovate-lanceolate, obovate-lanceolate, or almost lanceolate or circular in outline, 1–2.5 times as long as broad in our material. In contour, the dorsal surface is convex, often with some irregularities. The ventral surface is similar, except that a shallow longitudinal furrow extends from the opening of the triangular cavity to the posterior region (Fig. 2). This furrow may be lacking in the more flattened and nearly circular types, as also in the extremely lanceolate types. In side view, the organism appears lanceolate to linear-lanceolate, with the widest part near the anterior end. Material examined an

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Explanation Plate I

Fig. 1. Vegetative cell of *Gonyostomum Semen*, ventral surface, showing the two flagella, the chromatophores, the triangular cavity, the trichocyst-like rods, and the position of the nucleus. × 944.

Fig. 2. Diagrammatic representation of side and ventral views of the vegetative cell. The ventral groove and positions of flagella are indicated. × 944.

Fig. 3. Diagrammatic drawing of a cell mounted in 1 per cent acid fuchsin, showing the slime threads. × 377.

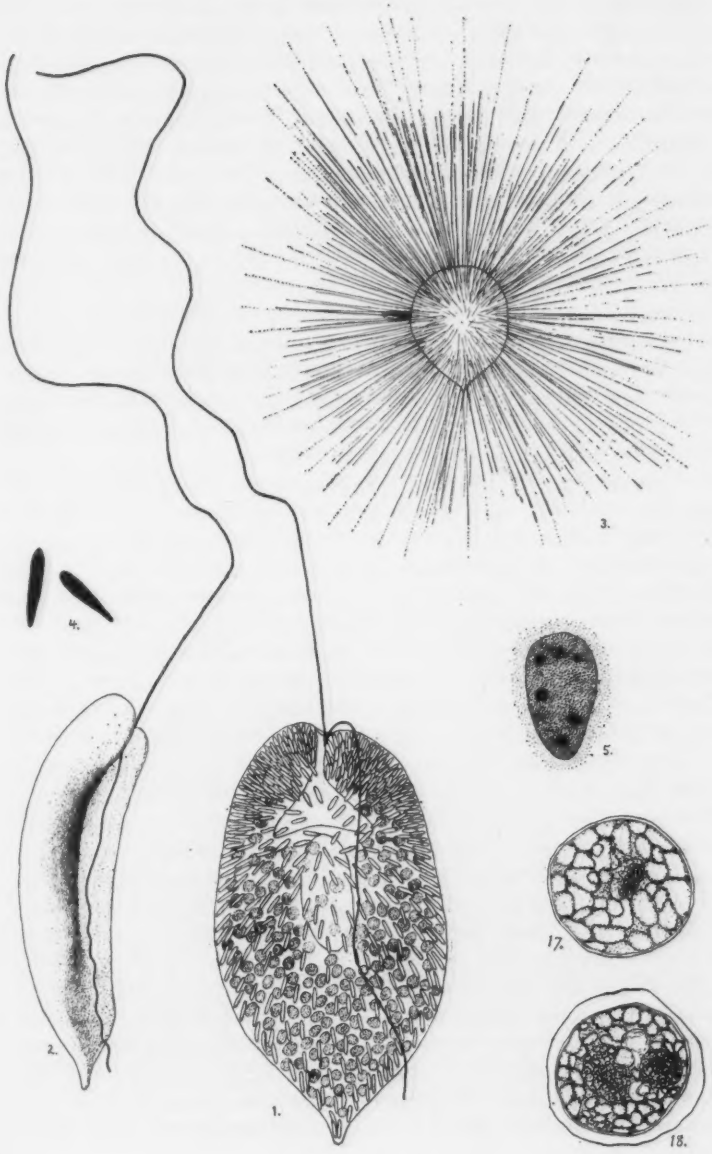
Fig. 4. Trichocyst-like rods discharged into the medium when the cell is mounted in very dilute aqueous acid fuchsin. × 2833.

Fig. 5. Nucleus as stained with cotton blue by Maneval's technique. The large endosome-like bodies and deeply-staining granular matrix are shown. × 944.

Fig. 17. Young cyst, showing the thin gelatinous sheath. × 708.

Fig. 18. Older cyst, with thick sheath, few chromatophores, and much oil. × 708.

PLATE I



hour after collection from the Cedar Swamp exhibited as a rule an abundance of the more lanceolate types (as seen in dorsal view); after a day or two of standing in the swamp water cultures in the laboratory, the organisms became more flattened and consequently broader in dorsal outline, so that almost circular cells were often observed; and the lanceolate types were indeed rare. In dorsal view, as also in ventral view, the anterior end is somewhat two-lipped, with the opening of the triangular cavity between the lips. Levander notes that the left lip (as seen in ventral view) is higher than the right lip, an observation which holds true in our material. Under certain external conditions, the caudus disappears entirely; and an organism the shape of *Vacuolaria virescens* results. Under pressure, or in viscid media such as 0.5 per cent agar or quince jelly as prepared by Turner (1917), the organisms become amœboid (Fig. 6). From the above description, it is obvious that the cell has no definitely fixed shape as has *Chlamydomonas* or *Peridinium;* the organism may be described as metabolic, with an ability to change shape in response to a change in the environment similar to that of *Euglena* or *Peranema*.

Ehrenberg's original diagnosis of *Monas Semen* describes the organism as having a length of 1/48 line (*ca.* 45 μ). Levander cites the length as 80 μ and the greatest breadth as 34 μ. Stein, and subsequently Kent, Lemmermann, and Lindau and Melchior (1926) say 44–63.5 μ. In one hundred random measurements of individuals made on various cultures during our observations, we have found that the size varies far beyond the above described limits. The largest individuals observed in our material measured 92 $\mu \times 69$ μ; the smallest 36 $\mu \times 23$ μ. Among these hundred cells, approximately three-fourths of the measurements lay between 40–80 $\mu \times$ 30–60 μ. Of the entire number the average length was 62.5 μ and the average width 41.4 μ. It is probable that under environmental conditions other than those to which the cells were subjected by us the extremes of size may be greater than our measurements indicate.

The nucleus, though obscured by other cell contents in the living cell, is easily observed, when the organism bursts, as an ovoid or subglobose body about one-fourth the length of the cell. It is found (in stained preparations) in the center of the cell, though sometimes to one side of the center or definitely in the posterior region. Stein figures the nucleus as containing a single deeply-staining body (the endosome) in its center surrounded by a well-delimited vesicle containing granular chromatic material. Levander states that the nucleus contains such chromatic filamentous structures as are seen in *Peridinium*. Our material, killed in 0.5 per cent osmic acid, preserved in

2 per cent formalin solution, fixed in very dilute chromo-acetic acid, and stained in Delafield's hæmatoxylin, shows the nucleus as a well-delimited vesicular body containing several to many almost spherical, deeply-staining bodies, each 1.5 μ or less in diameter, distributed through a coarsely granular matrix. No one or two of these can be distinguished from the rest as true endosomes, such as Stein figures for *Gonyostomum Semen* and Bütschli describes in *Vacuolaria virescens*. Slides prepared by Dr. W. E. Maneval from the material fixed in osmic acid and preserved in formalin, and stained as temporary mounts [2] with cotton blue or as semi-permanent mounts with a mixture of cotton blue and acid fuchsin, demonstrate this structure even better (Fig. 5). It is not improbable, however, that the formalin produces anomalous granules in the nucleus. In material which is dried on the slide before staining, the detailed structure of the nucleus is often obscured by the overlying chromatophores and trichocyst-like rods. No evidence of a filamentous structure of the nucleus was seen. No material was sectioned and stained.

The cytoplasm, as seen in the living cell, is hyaline, finely granular, and without evident vacuolar structure. In the periphery of the cytoplasm and sometimes throughout the mass, lie the contractile vacuoles, the chromatophores, and the trichocyst-like rods. The outer layers, in the living state, exhibit no conspicuous alveolar structure as that which Senn describes and figures in *Vacuolaria virescens*. Yet, when a cell bursts or is stained without preliminary drying, as with Maneval's stains described above, the cytoplasm in which the chromatophores etc. are embedded appears less viscous and less granular than that immediately surrounding the nucleus.

At the anterior end of the organism, a duct opening to the outside leads into a cavity in the cytoplasm usually described as triangular in optical longitudinal section (the 'dreieckige Blase' of Lemmermann and of Pascher). Oltmanns (1922) describes this cavity as "von der Form eines breiten Kegels (sie erscheint im Schnitt dreiseitig)." In our material the cavities are somewhat flattened dorsoventrally and vary in shape from one-third as wide as long (in ventral view of the cell) to twice as wide as long. The function of this cavity is not as

[2] The material is mounted directly in the following solution (Maneval): 15 parts of 5 per cent phenol, 4 parts of glacial acetic acid, and 1–3 parts of a 1 per cent aqueous solution of cotton blue. For the semi-permanent mounts, a modification of Amann's lacto-phenol (Maneval) was used: 20 grams of phenol, 20 cc. of lactic acid, and 40 cc. of glycerine are dissolved in 20 cc. of water. To this 20 per cent by volume of glacial acetic acid is added. Finally, equal parts of 1 per cent aqueous cotton blue and 1 per cent aqueous acid fuchsin are added to the mixture in quantities suitable for the type of staining preferred.

yet clearly understood. Morphologically, the triangular cavities of *Gonyostomum Semen* and of *Vacuolaria flagellata* (Stokes) Senn (Stokes, 1886 and 1888; Senn, 1900) may be homologous with the so-called 'flagellar pits' of *Thaumatomastix setifera* Lauterb. and of *Vacuolaria virescens* Cienk. (Lauterborn, 1899; Cienkowsky, 1870; Bütschli, 1883; Senn, 1900). In the works on the flagellates cited at the end of this paper, one is given the impression that the contractile vacuoles discharge into this cavity.[3] Various observations of our own, insufficient at present for definite conclusions, indicate that this supposition is plausible. However, other observations indicate that the contractile vacuoles discharge through the outer cell membrane. The development of triangular cavities in dividing cells is described below.

One large contractile vacuole, said by Levander to contract about once every minute, is present in the cytoplasm somewhere in the vicinity of the triangular cavity. Levander places it "am Ende des Geisselkanals," though in our material it is more often found nearer the outer cell membrane than the cavity. Smaller contractile vacuoles were observed in some individuals scattered through the anterior cytoplasm. These appear to coalesce sooner or later and finally to empty into the large anterior vacuole. Whether the large vacuole discharges at length through the outer cell membrane or into the triangular cavity, we cannot at present be certain.

The chromatophores are small ovoid bodies of a peculiarly bright green color—the *maigrün* of German authors. As in other chloromonads, the chromatophores of *Gonyostomum Semen* become a dull blue-green in color when treated with a dilute acid, *e.g.*, 5 per cent HCl, a reaction (Pascher, Senn) indicative of the presence of an excess of xanthophyll. The chlorophyll (Pascher) is thought to be of a chemical composition somewhat different from that encountered in other flagellates and green plants. The chromatophores are arranged peripherally in the cytoplasm over the entire body of the organism with the exception of the caudus. Red-pigmented bodies (eye-spots, stigmata) are apparently absent.

No starch or starch-like substances which produce a blue color

[3] Oltmanns speaks of the function of the cavity: "Sie ist selber nicht kontraktil, steht aber mit pulsierenden Vakuolen in Verbindung, die wohl in sie einmünden. Die Sache erinnert an Euglenen und an Peridineen." Likewise, Senn remarks: "Bei anderen Formen . . . (Rhaphidomonas und Thaumatomastix) hat sich eine constant vorhandene, nach aussen offene, nicht mehr pulsierende Hauptvacuole ausgebildet, in welche sich die seitlich entstehenden Nebenvacuolen abwechselnd entleeren (Fig. 125)." The figure cited illustrates the vacuolar system of *Thaumatomastix*, redrawn from Lauterborn.

when treated with iodine were found in the cells. Oil occurred as slightly amber-colored globules distributed through the cytoplasm of cells observed shortly after collection from the Cedar Swamp. As the laboratory cultures became older, the oil droplets grew larger and more conspicuous, especially in the broader and more flattened individuals. Often one or two large irregular oil bodies comprised half the length of the cell and measured one-third to one-half as wide as long in optical section. Since these conspicuous droplets were not seen in small individuals which showed evidence of recent or future division, it is supposed that they indicate a pathological condition of the protoplasm. Similar production of excess oil under unfavorable cultural conditions was observed by Bohlin (1897) in *Chloramœba heteromorpha*. Treatment with 0.5 per cent osmic acid for a week or more at room temperature blackens the oil droplets entirely. A saturated alcoholic solution of Sudan III causes the cells to burst and allows the droplets to lie free in the medium; these droplets absorb the Sudan III readily. When organisms killed in the fumes of osmic acid are allowed to dry on the slide, the oil globules are easily discerned in the dried cells. If a drop of xylene or Canada balsam is placed on the material, the oil globules disappear. Cells fixed by other agents likewise lose the oil droplets when mounted in xylene or balsam. Levander records the finding of paramylon [4] in the cells of *Gonyostomum Semen*. He gives us no reason to suppose that he investigated the chemical nature of these bodies in the painstaking manner employed by Bütschli (1906) on the paramylon bodies of certain Euglenophyceæ.

Two flagella are present, each as long as, or longer than, the body of the organism. The one projected forward (tractellum) is attached to the inner wall of the duct leading from the triangular cavity. The insertion of the trailing flagellum (pulsellum) has not been determined; however, if it were inserted on the wall of the duct its basal portion would surely have been seen there. It is probably attached to the cell membrane near the anterior end of the ventral groove, as Stokes has recorded in *Vacuolaria flagellata*. As Levander has already noted, the organism progresses forward in a slow, spiral course, the basal portion of the tractellum projected directly in advance, moving little if at all, the apical portion spiralling so rapidly that it is invisible until the organism has come to rest. Then, when the cell is quiescent, such as following the addition of very dilute acid fuchsin (1 drop of 1 per cent aqueous acid fuchsin in 50 cc. water) to the mount, both

[4] To quote (p. 34): "An der Dorsalseite des Vorderendes sah ich öfters einige ganz minimale, ringförmige Körner, die wohl Paramylum-körner waren."

flagella can be observed. The pulsellum was never seen in its entirety and is therefore shown in Fig. 1 as shorter than the tractellum. In the dilute acid fuchsin, the vibratile portion of the tractellum lashes back and forth in slow spirals; the pulsellum often leaves the ventral groove and is projected outward or forward. The attenuated apical portion of the tractellum was seen also by carefully focusing the oil-immersion objective when dark-field illumination was employed. Since the apical parts of the flagella are so delicate, an almost perfect dark field, free from foreign particles in the mount and from scratches on the glassware, is necessary. It was found impracticable to stain the flagella, since the gelatinous threads discharged from the trichocyst-like rods obscure the flagella when a stain is applied. Since no microtome sections were made, basal granules and other structures of a kinetic apparatus remain unidentified.

No cell wall is present about the organism. The cell has, as a rule, a definite shape as has *Euglena;* only under certain marked departures from the usual conditions found in the medium, however, does the shape change as rapidly as does that of *Euglena*. The protoplast itself is delimited from the surrounding medium by a very thin plasma membrane, which does not separate from the rest of the cytoplasm when the organism is placed in concentrated salt solutions like chlorzinc-iodine (Nowopokrowsky, 1911), as does a cell wall. No blue or violet color appeared during treatment with this reagent to indicate the presence of cellulose or related carbohydrates in the membrane. When the liquid beneath the cover-slip dries slowly so that the organisms are subjected to gradually increasing pressures, the cells become amœboid. Under such pressures, a definite and distinct line (or membrane) can be seen delimiting the outer cytoplasm of each lobe. Sooner or later, if the pressure continues to be increased, the membrane breaks; and the organism bursts beyond recognition. While the membrane is still intact, the flagella move slowly or remain stationary; if more water is added to the mount (and the pressure thereby diminished), the cell regains its usual shape and the flagella resume normal activity. Amœboid cells of *Gonyostomum Semen* were found in a medium containing 0.5 per cent agar in swamp water. In such cultures, the cells lived at least 24 hours with the chromatophores and other cell structures in a healthy condition and with the trichocyst-like bodies undischarged. The lobed condition here is due probably to mechanical pressure exerted by the agar on the surfaces of the cells. Amœboid shapes were likewise assumed by cells in the process of division, as described below. The fact that *Gonyostomum Semen* exhibits such 'metaboly' as do various of the Euglenophyceæ

affords further evidence, though indirect, of the absence of a true cell wall.

Distributed radially in the peripheral cytoplasm are rod-shaped bodies (Fig. 1) characterized in the living cell by their ability to absorb large amounts of dye from very dilute aqueous solutions. Ruthenium red, Gram's iodine, acid fuchsin, methylene blue, methyl green, safranin, and Delafield's hæmatoxylin are a few of the dyes absorbed in this manner. As Levander has stated, the rods are arranged in dense 'palisade-like' formation in the anterior half of the cell, and here are most abundant in the lobes on either side of the triangular cavity. Toward the posterior end the distribution is less orderly and less abundant. The objects appear to be least numerous in the caudus, though some individuals have been observed to possess as many as ten of the rod-like bodies here.

When a very dilute solution of a dye (1 drop of 1 per cent aqueous acid fuchsin in 50 cc. water) is added to the mount, a few of the rods burst suddenly through the cell membrane and become elongated into threads of slime as long as or several times longer than the body of the organism. Others come through the cell membrane and lie free in the medium as club-shaped bodies (Fig. 4), rounded at one end and pointed at the other. Others remain half on either side of the membrane, but most remain in their original positions within the cytoplasm. In any case, the bodies quickly absorb the dissolved dye, whether they are inside or outside the cell. When more concentrated solutions of dyes (such as 1 per cent aqueous acid fuchsin) are allowed to diffuse beneath the cover-slip, the rods are discharged suddenly, and the slime-threads produced become deeply stained at once, so that the cell becomes the center of a mass of radiating gelatinous threads (Fig. 3). The threads often appear branched and *en masse* are similar to a much-branched fungus mycelium surrounding the cell, as described by Levander. When discharged in a salt solution, without the addition of a dye, the threads are difficult to see except at their bases, where they have the greatest diameter when stained. If the cells are mounted in chlor-zinc-iodine, the rods do not discharge, as Scherffel (1912) has described in *Monomastix* and *Pleuromastix*, nor do they leave the cell without elongating, as Levander has described in *Gymnodinium*.

A large percentage of the cells treated with 1 per cent solutions of the dyes mentioned above burst when the trichocyst-like bodies are suddenly discharged. It is supposed here that the membrane surrounding the protoplast is broken as the rods or filaments shoot through it. Scherffel has described and figured visible holes in the cell mem-

branes of *Monomastix*, but the rods in that organism are comparatively much larger and less numerous than those of *Gonyostomum*. Often the discharge of the bodies and consequent bursting of the membrane (or *vice versa*) can be caused by pressure. This is demonstrated when a thin film of culture medium containing the cells dries on the slide. The gradually increasing concentration of dissolved materials in the medium may be partially responsible for the bursting or discharge, but similar results are obtained when the organisms dry in distilled water.

The bursting of the cells upon the addition of various dissolved materials introduces the greatest difficulty in handling *Gonyostomum Semen*. Several types of killing and fixing agents were used on the cells with disastrous results: 1 per cent–95 per cent alcohol, $HgCl_2$ (concentrated solution in water and in 50 per cent alcohol), chromo-acetic acid (1–10 drops in 20 cc. water), Flemming's solution, and 1 per cent–3 per cent formalin in water. Inverting a slide containing a drop of the culture solution over the fumes of 1 per cent osmic acid met with the best success. The organisms also were killed satisfactorily when an equal part of 1 per cent osmic acid was added to the medium. Maneval's nuclear stain [5] proved almost as successful as a killing agent. If first killed in osmic acid or Maneval's stain, the cells could be fixed in other reagents without distortion or bursting. Difficulties similar to those encountered in fixing and staining were met with also in transferring from one culture medium to another.

When a collection is taken from the Cedar Swamp and left standing in a jar of swamp water for a day, many of the organisms fall to the bottom of the jar and become aggregated there in a gelatinous stratum. A piece of such a mass under the microscope appears to be composed of cells of the usual vegetative structure lying in a gelatinous matrix. When stained with very dilute iodine or acid fuchsin, the gelatinous material is seen to be composed of mycelium-like threads such as those about organisms from which the trichocyst-like bodies have been discharged because of a chemical stimulus. The cells, upon close inspection, contain few of the rod-like bodies. It is probable that the rods are discharged because of some change in the chemical or physical nature of the medium; the slime-threads of various individuals become

[5] A variation of the first stain described in footnote 2 of this paper: 60 parts of 5 per cent aqueous phenol, 10 parts of 30 per cent aqueous $FeCl_3$, 20 parts of glacial acetic acid, and 15 parts of 1 per cent aqueous acid fuchsin. The organisms are mounted directly in this solution. If the staining is too deep, the material may be destained to the desired color with Amann's lacto-phenol (the second solution of the same footnote without the dye added).

enmeshed; the flagella are caught in the gelatinous mass and are rendered functionless; and the organisms sink to the bottom of the jar because of their specific gravity. In the gelatinous mass the penetration of osmic acid and stains is very slow. In the case of stains, at least, the dissolved material is absorbed in large quantities by the outer part of the matrix. Senn (p. 104) gives us the impression that such gelatinous coverings are produced by many flagellates: "Gelegentliche Ausscheidung weicher Gallerte ist bei sehr vielen, besonders mit Chromatophoren versehenen Formen (Euglenaceae, Chloro- und Chrysomonadineae) haüfig. Durch ungünstige Verhältnisse (Druck, Zusätze von Reagenzien) treten aus dem Periplasten geschlangelte Gallertfaden, die durch ihre nachträgliche Verquellung die Zelle in einen losen Mantel einhüllen. Mit dieser gelegentlichen Gallertausscheidung muss auch die Bildung von Gallerthüllen durch Dauercysten in Beziehung gebracht werden." In *Euglena*, Klebs (1883) described the slime-producing bodies as being attached to the cell membrane. In *Gonyostomum Semen*, the bodies do not appear to be attached to the membrane nor do they appear to be attached to the membrane in Levander's figures of *Gymnodinium* or in Scherffel's figures of *Monomastix* and *Pleuromastix*. Whether or not these bodies in the green flagellates can be viewed as true trichocysts such as those described by Mitrophanow (1905) and Schuberg (1905) in *Paramœcium* and other Ciliata remains an open question.

Cell Division

Many of the smaller individuals found in the free-swimming and the gelatinous conditions described above, upon being treated with nuclear stains, showed the presence of two nuclei within each cell (Fig. 7). Other individuals exhibited 'swallow-tails' (Figs. 8 and 9) at the posterior ends. These peculiar features appeared in the cultures regularly during midday and the early evening. They led us to look further into the matter of cell division.

Hanging-drop cultures of swamp water were constructed and observations made almost continuously during the periods 11:00 A.M. to 1:00 P.M. and 6:30 P.M. to 8:30 P.M. Standard Time. A large number of cells were seen in various stages of division, and the entire process was watched in several individuals. The time required for complete division in the swamp water cultures was regularly 45–55 minutes. If an equal quantity of quince-seed jelly were added to the hanging-drop, the movements were slowed down and the time required for the process lengthened.

Division is longitudinal, as in other wall-less Flagellata, often

slightly oblique. The cell enlarges in transverse section, becomes more metabolic and amœboid in form, and loses the flattened shape so characteristic of the vegetative individual. Usually a lobe appears at one side of the caudus, so that two 'tails' are apparent. From each arm of the triangular cavity, as seen in ventral optical section, an extension of the cavity is sent posteriorly and laterally (Fig. 9). At the extremities of these arms, two new arms are pushed out at angles to each other into the cytoplasm (Fig. 11). These enlarge to form two new triangular cavities, each opening into the original one. Accompanying or following the formation of new cavities, a longitudinal furrow extends about the long axis of the cell body, beginning with the constriction between the 'tails' or at the anterior end in a plane bisecting the original triangular cavity longitudinally (Figs. 12–15). No matter at which end it is first evident, the furrow soon becomes conspicuous at both ends and extends rapidly to the central part of the body, ultimately constricting the mother into two daughter cells (Fig. 16). The flagella move vigorously throughout this process, and the entire body of the cell becomes contorted, exhibiting much 'metabolic' activity. New flagella appear soon after the anterior end splits in division (Fig. 14), so that the dividing individual possesses four wildly lashing flagella, seemingly hastening the separation of the cells by pulling in different directions. The new triangular cavities, as may be readily seen in Figs. 11, 12, 13, and 15, are not formed originally at the anterior apices of the constricting cells; rather, they

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EXPLANATION OF PLATE II

FIG. 6. Amœboid cell body produced by pressure of the cover-slip in a gradually drying mount. × 708.

FIG. 7. Two-nucleate cell found in swamp water culture at noon. Fixed in osmic acid and stained with Delafield's hæmatoxylin. × 377.

FIG. 8. Cell in the first stages of division, with 'swallow-tails' at the posterior end. × 377.

FIG. 9. Cell with elongated and 'lobed' triangular cavity and with conspicuous 'swallow-tails.' × 472.

FIG. 10. Cell with first constriction appearing in the anterior region, instead of in the posterior as in Figs. 8 and 9. × 472.

FIG. 11. Amœboid cell in division, showing the triangular cavities of the daughter cells formed at the base of the cavity of the mother cell. × 708.

FIG. 12. The same cell constricting at the anterior end. This constriction appears to bisect the triangular cavity of the mother cell. × 708.

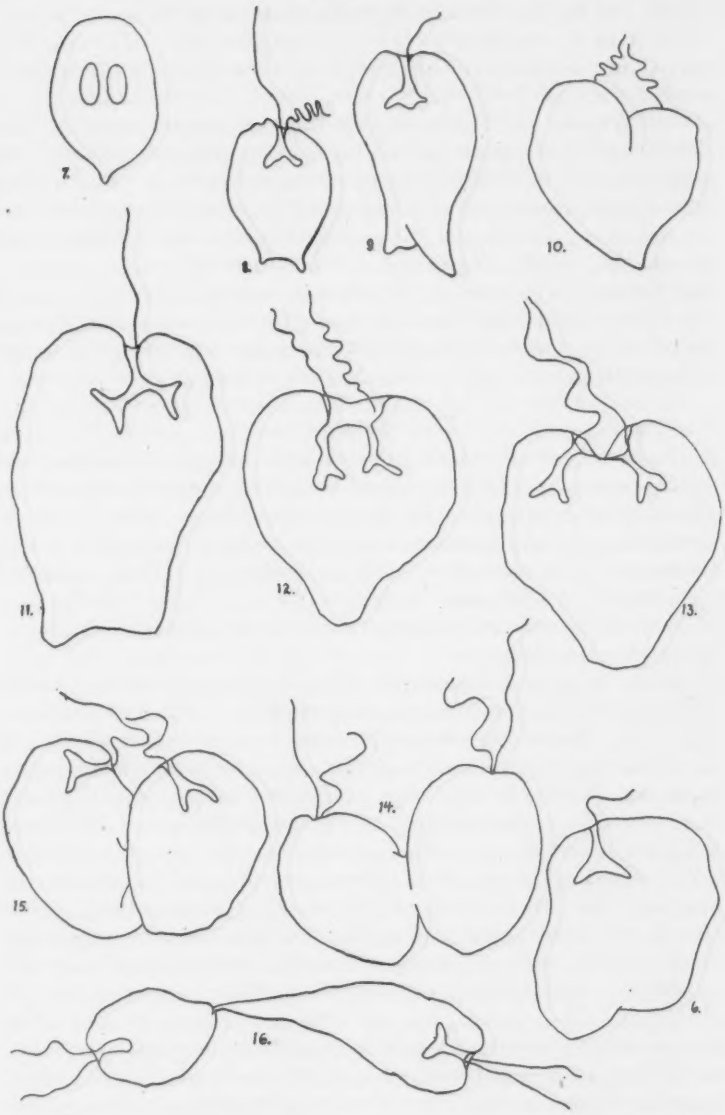
FIG. 13. A later stage in constriction of the same cell. × 708.

FIG. 14. The two daughter cells attached by only a small protoplasmic connection, each cell bearing two flagella. × 708.

FIG. 15. Same as Fig. 14, but showing the two cells with distinct triangular cavities. × 708.

FIG. 16. The same, with the daughter cells pulling apart. × 708.

PLATE II



first appear laterally, and by the splitting of the original triangular cavity and by change in shape of the daughter during the later stages of division, are finally (Fig. 16) oriented at the apex. The fate of the flagella of the mother cell and the origin of the flagella of the daughters were not noted.

As pointed out above, the dividing cells were primarily those belonging to the smaller size ranges of the population, though a few large flat cells appearing as the culture aged exhibited 'swallow-tails.' These latter types invariably contained large irregular oil bodies and possibly were pathological, for none were observed to proceed further in division. Such observations, if simulated by future studies in *Gonyostomum Semen*, may provide further evidence in favor of Adolph's (1931) contention that the frequency of division is inversely proportional to protoplasmic growth; in other words, the larger the cells in the population, the smaller the frequency of division.

Encystment

Encysted cells appeared in two-day-old hanging-drop cultures of swamp water and also in tightly stoppered swamp-water cultures placed for several days in the dark. In the latter cultures, the encysted material was abundant and lay in yellowish palmelloid masses in the bottom of the liquid. Cultural conditions in these stoppered dishes were obviously unfavorable, for the cysts here exhibited signs of deterioration—the production of much oil and evident disintegration of the chromatophores.

In the hanging-drop cultures, young cysts appeared as spherical bodies 25–36 μ in diameter and surrounded by a thin hyaline sheath (Fig. 17). In some specimens the caudus could be distinguished as an actual projection of the spherical body until the sheath had grown to several microns in thickness. The older cysts (Fig. 18) have a thick gelatinous sheath like that which Cienkowsky (1870) has described and figured for *Vacuolaria virescens*. No trichocyst-like bodies were apparent in these older cysts. Beneath the sheath, the peripheral layer of chromatophores, many oil globules, and several (1–3 in our observations) contractile vacuoles were distinguished. These vacuoles, each measuring 3–4 μ in the diastole, were observed, especially in the young cysts, to discharge directly through the cell membrane. The nucleus was not identified. In old cysts, large clumps of dark brown material appeared in the cytoplasm. The sheath stains intensively with ruthenium red and other dyes, a characteristic which leads us to suspect that pectic substances are present. The sheath obviously prevents the ready passage of dissolved dyes (and other substances?) into the included protoplast.

Division probably occurs in these encysted cells. Our limited time did not permit such observations on this material.

Summary

1. The chloromonad, *Gonyostomum Semen* (Ehrenb.) Diesing, was collected in a sphagnum swamp at Woods Hole, Massachusetts, during July, 1934. The organism has been seen so rarely since it was first described that certain morphological features of the vegetative cells have been imperfectly known, and cell division and encystment have not previously been recorded.

2. The vegetative cells are flattened dorsoventrally and vary in shape in ventral view from lanceolate to circular. The anterior end is two-lobed, and a short caudus terminates the posterior end. No cell wall is present: the cells are somewhat metabolic and under certain conditions become amœboid. The extreme size measurements of a hundred cells were 36–92 $\mu \times$ 23–69 μ. On the ventral surface, a shallow longitudinal groove runs from anterior to posterior regions. Two flagella are present, one projected forward, and one trailing close to the cell body in the ventral groove. A broadly conical cavity lies in the anterior cytoplasm and opens to the outside by a small aperture between the anterior lobes. A large contractile vacuole is seen near this cavity, and several smaller ones may be present. The nucleus is ovoid or subspherical and usually located centrally in the cell. Its structure has not yet been clearly shown. The chromatophores are small, ovoid, and arranged peripherally in the cytoplasm. Oil is the product of metabolism, and under obviously unfavorable conditions it is formed in large quantities. Many hyaline rod-like bodies are arranged radially in a peripheral layer in the cytoplasm. These bodies absorb large amounts of stains from very dilute solutions. Under pressure, or upon addition of chemicals, these bodies shoot out of the cell and usually elongate into threads of slime. They act like the trichocysts in *Paramœcium* and force the worker to exercise extreme care in fixing, preserving, and culturing the organisms.

3. Division of vegetative cells is longitudinal; it occurs, as a rule, only among the smaller cells of the population. Nuclear division and cytoplasmic constriction are described.

4. Encystment of cells was observed in old cultures. Division of encysted cells was not observed.

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COMPARISON OF THE REACTIONS AGAINST HETEROTRANSPLANTED TISSUES IN DIFFERENT KINDS OF HOSTS[1]

LEO LOEB

(From the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri)

In several preceding papers, we have studied the reaction of organisms against heterotransplanted tissues, in particular as to the correspondence between these reactions and the phylogenetic relationships between host and transplants.[2] On the whole, we found a lack of such a correspondence, if we used mammalian and avian organisms as hosts, although it is present in more primitive classes of animals. However, even in mammalian hosts or transplants there seemed to be found at least an indication of such a correspondence; but this was not definite. We wished therefore to investigate this question again; comparing the behavior of two very nearly related species, such as rat and mouse, with more distant species, such as guinea pig and rat, cat and rat, pigeons and rat. We also compared in these investigations again some reciprocal transplantations, in order to determine on what factors the difference in the results depended; and lastly, we wished to study once more some of those modes of reactions which distinguished heterotransplantation from homoiotransplantation.

From a different point of view, namely, in their relation to strain or race transplants, we have considered already certain general aspects of transplantations from mouse to rat and the reciprocal transplantations.[3] We shall now consider these transplantations in their relation to heterotransplantations between further distant species, and we shall also compare the action of various tissues and cells of the hosts on these transplants. As usual, all our transplants were inserted into pockets in the subcutaneous tissue of the abdominal wall, and they were examined at different periods following transplantation. We

[1] These investigations were carried out with the aid of a grant for research in science made to Washington University by the Rockefeller Foundation.

[2] Loeb, Leo, and W. H. F. Addison. *Arch. f. Entw.-mech.*, **27**: 73 (1909) and **32**: 44 (1911).

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[3] Loeb, Leo and Helen D. King. *Am. Nat.*, **69**: 5 (1935).

used either thyroid and cartilage, together with the adjoining tissues, or cartilage with the adjoining tissues alone. In a few cases we grafted also the ovary. The transplants of thyroid and ovary were cut into complete serial sections and, in the case of the cartilage transplant, sections through the different parts of the transplant were examined. Altogether one hundred and thirty-four pieces were studied in this manner. We shall cite summaries of our findings at eight to ten and at twenty to thirty days following transplantations.

Eight to Ten Days after Transplantation

From rat to mouse and reciprocal transplantations.—In the rat to mouse transplants of thyroid, the grafted tissue has become necrotic; only in one specimen (after 8 days) were there still some acini with colloid preserved. Also muscle tissue transplanted with thyroid was necrotic. Fat tissue was largely necrotic and much infiltrated with connective tissue; a great many polymorphonuclear leucocytes were found especially in the necrotic areas. The surrounding connective tissue capsule was infiltrated with lymphocytes.

In the mouse to rat transplants of thyroid, the graft has been replaced by fibrous tissue, which is densely infiltrated with lymphocytes. In one specimen, there were some indistinct remnants of thyroid tissue seen. The transplanted ovaries were necrotic and, in the periphery, invaded by connective tissue, which transformed the transplant into a loosely vacuolar tissue. There was also noticeable some infiltration with lymphocytes.

In the rat to mouse transplants of cartilage, the cartilage and perichondrium were, in some cases, mostly preserved; in other cases, necrotic. Intermediate conditions were also found. The extent of infiltration of the transplant with polymorphonuclear leucocytes varied in different specimens; they were found especially in the necrotic fat tissue. There was also some definite lymphocytic infiltration, but it was not so marked as at later periods. The lymphocytes were found mostly in the distant connective tissue surrounding the transplants. The fat tissue was largely necrotic, especially near necrotic perichondrium.

In the mouse to rat transplants of cartilage, there was, on the average, a better preservation than in the reciprocal transplants. The perichondrium was partly replaced by lymphocytes, which penetrated a little into cartilage. Cartilage and perichondrium were surrounded by fibrous tissue densely infiltrated with lymphocytes. Necrotic cartilage was also invaded by connective tissue cells. The transplanted muscle tissue was necrotic. Bone and bone marrow were necrotic

and the bone marrow was partly or completely replaced by connective tissue; there was a dense lymphocytic infiltration around the bone. After eight days, there was some infiltration with polymorphonuclear leucocytes, but after ten days, these cells had disappeared. In the fat tissue some hyperemic and hemorrhagic areas were found. In no case were there any regenerative processes seen in cartilage and muscle tissue. In specimens examined at six days transplanted cartilage and fat tissue were still preserved, as were acini of the thyroid at this time. There were large hemorrhagic areas in fat tissue. Again there was more fibrous tissue formation and lymphocytic infiltration in the mouse to rat than in the reciprocal transplantations. While there was here some infiltration with polymorphonuclear leucocytes, the maximum was reached after eight days.

From rat to guinea pig and reciprocal transplantations.—The results in the transplantations of thyroid from rat to guinea pig were very similar to those obtained after transplantation of this tissue from rat to mouse. In general, we found the transplanted acini as necrotic material in the center of the transplant. There was much infiltration with polymorphonuclear leucocytes in the dead tissue after eight days. In the peripheral tissue we found very moderate lymphocytic infiltration. In one specimen after eight days, there were, in the periphery of the transplant, some isolated acini with solid colloid in the connective tissue and in another specimen the outer epithelial walls of a few acini were preserved. Also after ten days, we found in one specimen a few peripheral parts of the walls preserved. The necrotic thyroid and fat tissue were still surrounded by a mantle of polymorphonuclear leucocytes, but in the surrounding connective tissue there was now more lymphocytic infiltration.

In the reciprocal transplants from guinea pig to rat there was, after eight days, some necrotic and hemorrhagic material being organized by fibroblasts and partly in process of hyalinization. There was a certain amount of diffuse lymphocytic infiltration, which was rather dense in some areas of connective and fat tissue. The infiltration with polymorphonuclear leucocytes was less in this case than in the reciprocal transplants. Especially in the ten-day transplants, the amount of necrotic thyroid tissue and the number of polymorphonuclear leucocytes were less than in the reciprocal transplants; but there was more lymphocytic infiltration and there was also a greater activity of phagocytes than in the reciprocal transplants.

In the eight-day cartilage transplants, we find some variations as to preservation of cartilage in different specimens, the preservation being, on the whole, greater in the guinea pig to rat than in the re-

ciprocal transplants. Polymorphonuclear leucocytes moved into the necrotic fat and also into the necrotic cartilage tissue There were some remnants of hemorrhage in the fat tissue; there were also lymphocytes and connective tissue in the interstices between fat cells. Muscle tissue and bone marrow were necrotic or the necrotic tissue was replaced by fibrillar connective tissue. In the guinea pig to rat transplants the fat tissue was mostly replaced by fibrous tissue. Here, as in the mouse to rat transplants, the lymphocytes were more active; they infiltrated the connective tissue around bone and cartilage and penetrated a little way into the perichondrium. Again, similarly to the mouse to rat transplants, there was here more fibrous tissue formation, a more marked infiltration with lymphocytes, and less infiltration with polymorphonuclear leucocytes. After ten days, more connective tissue and lymphocytes and fewer polymorphonuclear leucocytes penetrated into the necrotic cartilage and bone than in the rat to guinea pig transplants. Lymphocytes moved also into cartilage and bone which they helped to destroy and these cells could replace fat tissue. As in the mouse to rat transplants, there was more connective tissue and lymphocytic activity and less necrosis in the guinea pig to rat than in the reciprocal transplants.

Transplantation of cartilage from cat to rat.—Cartilage could be preserved. Necrotic muscle and bone were replaced by connective tissue. Fat tissue was partly necrotic, partly replaced by connective tissue; the infiltration with cells of a different nature made it difficult to determine whether some fat cells were still alive. Much dense lymphocytic infiltration was found in surrounding tissue. In these transplants polymorphonuclear leucocytes were not as prominent as in the rat to mouse transplants. The lymphocytic infiltration around cartilage and in muscle was less marked than in transplants from mouse to rat. No regeneration was observed in perichondrium or muscle tissue.

Transplantation of cartilage from rat to pigeon.—Cartilage and perichondrium were either necrotic or parts of it, varying in amount in different cases, were preserved. The other transplanted tissues, including the fat tissue, were necrotic. Hemorrhages were found in the fat tissue and the latter was invaded by polymorphonuclear leucocytes, which moved also into the necrotic cartilage and muscle tissue. There were some phagocytic cells between the fat cells. Lymphocytic infiltration was still slight at this time.

Twenty to Thirty Days after Transplantation

Mouse to rat and reciprocal transplantations.—The transplanted thyroid had in all cases been replaced by fibrous tissue with lympho-

cytic infiltration; but in the mouse to rat transplantations, the lymphocytic infiltration was, as usually, much denser than in the reciprocal transplantations. In the rat to mouse transplants, on the other hand, a few polymorphonuclear leucocytes were still seen. The transplanted pieces of ovary were necrotic and surrounded by lymphocytes. Cartilage and perichondrium were entirely necrotic in the rat to mouse transplantations, but in about one-half of the specimens transplanted from mouse to rat the greater part of these tissues were preserved, while the other pieces were entirely necrotic. In both transplants lymphocytes penetrated into, destroyed, and partially replaced necrotic cartilage and perichondrium; also connective tissue penetrated into necrotic cartilage. On the other hand, these cells did not seem to invade preserved transplanted cartilage. The transplanted fat tissue was either replaced by masses of lymphocytes or connective tissue and when the fat cells were still preserved, lymphocytes, connective tissue, and vacuolated phagocytic cells had penetrated into the interstices between the fat cells and separated them. Transplanted muscle tissue, bone, and bone marrow were necrotic; bone marrow had been replaced by connective tissue and lymphocytes. Connective tissue grew also into the dead bone, around which giant cells had formed. The formation of a fibrous capsule, as well as lymphocytic infiltration, was more marked in the mouse to rat than in the reciprocal transplants; this accords with what we found also in earlier stages. Especially the lymphocytic infiltration was more dense in the mouse to rat transplants; this is true too of the connective tissue around necrotic cartilage and bone. But also in the rat to mouse transplants there was occasionally a more marked lymphocytic infiltration around the cartilage. Polymorphonuclear leucocytes were found in the connective tissue surrounding necrotic fat tissue and they also penetrate into the latter; but these cells were not frequent at this period and they were especially rare in the mouse to rat transplants. We see then that between twenty and thirty days following transplantation almost all the tissues are necrotic or replaced by connective tissue and lymphocytes.

Guinea pig to rat and reciprocal transplantations.—After twenty days we still found necrotic material in the center of the thyroid transplant; it was surrounded by a connective tissue capsule which was much infiltrated with lymphocytes; there were among the latter also some polymorphonuclear leucocytes. Connective tissue cells slowly invaded and organized the central necrotic material. In these transplants, the invading connective tissue cells first converted the necrotic material into a hyaline fibrillar material; this represented the

first step in the organization. Cells with much cytoplasm then moved with pseudopods into the hyaline material; they enclosed particles of the latter in their cell body and digested them. Thus a vacuolated tissue developed. After twenty-five days the necrotic material had disappeared and only nodules of fibrillar connective tissue were left. These were infiltrated with lymphocytes. Some polymorphonuclear leucocytes were still present in certain nodules, probably left over from the former stage, when there was still necrotic material present.

Transplantation of cartilage.—At twenty days, cartilage and perichondrium were mostly necrotic; small parts were preserved in the rat to guinea pig transplants and somewhat more in the reciprocal transplants. Fat tissue and bone marrow were necrotic or replaced by fibrillar connective tissue; muscle tissue was necrotic. In the rat to guinea pig transplants some polymorphonuclear leucocytes moved into the necrotic cartilage and fat tissue. Lymphocytes infiltrated the fat tissue. In the guinea pig to rat transplants lymphocytic infiltration was found around the necrotic bone, connective tissue, and cartilage; lymphocytes penetrated also into the perichondrium. In these transplants there was more fibrous tissue formation, more lymphocytic infiltration, and less invasion by polymorphonuclear leucocytes than in the reciprocal transplants. After twenty-five days, the transplanted tissues were necrotic, except in one piece, in which in the guinea pig to rat transplant, cartilage cells were in some parts still preserved. Connective tissue cells with many lymphocytes invaded the necrotic cartilage. There were also many polymorphonuclear leucocytes, in certain cases, around the necrotic cartilage. Fat tissue was either replaced by fibrillar connective tissue or the septa between the fat cells were invaded by connective tissue cells and by cells which took up small fat droplets. In the connective tissue, which had replaced part of the fat tissue, polymorphonuclear leucocytes could be seen in some instances. In the necrotic fat tissue giant cells were found and giant cells formed also around necrotic cartilage and muscle tissue.

In transplants of cartilage from cat to rat after twenty days.—Parts of cartilage and perichondrium were preserved; other parts were necrotic. Around the cartilage the connective tissue was densely infiltrated with lymphocytes and from here lymphocytes invaded the cartilage and dissolved it. Fat tissue was mostly necrotic. In the fibrillar connective tissue surrounding it there was much lymphocytic infiltration and these lymphocytes and connective tissue cells pushed their way between fat cells; again we found here vacuolar phagocytic cells which had taken up fat droplets. The muscle tissue was necrotic and in process of organization by connective tissue. In the connective

tissue capsule surrounding the piece there was much lymphocytic infiltration. It may be stated—and this applies also to other cases in this series—that it is often difficult to determine whether or not small parts of the transplanted fat tissue are still alive, when so many cells coming from the host tissue push their way into the interstices between the fat cells.

Transplantation of cartilage from rat to pigeon; examination twenty and twenty-five days after transplantation.—After twenty-five days, the transplants were entirely necrotic, and giant cells were seen around the pieces. After twenty days, however, there were in certain areas of the cartilage some cells still preserved. In one place at the border of cartilage and perichondrium, the hyaline tissue was dissolved, owing to the taking up of fluid by this tissue, which thus bulged out. The cartilage cells were preserved and had cell processes in this soft material. In some specimens after twenty and twenty-five days strands of lymphocytes penetrated into the necrotic perichondrium or also into the cartilage and into interstices in the dense connective tissue surrounding the cartilage; with the lymphocytes, in some cases, polymorphonuclear leucocytes invaded the necrotic cartilage. The fat tissue was necrotic; it was either replaced by connective tissue or by a tissue consisting of vacuolated cells, which evidently had taken up fat droplets. Lymphocytes surrounded single and coalesced fat cells and furthermore collections of polymorphonuclear leucocytes were found in the fat tissue. The transplant was encircled by node-like collections of lymphocytes, between which a diffuse infiltration of lymphocytes was found in the small-celled, fibrillar connective tissue. Mitoses were occasionally seen in these lymphocytes. It was especially the necrotic material which attracted the polymorphonuclear leucocytes.

Discussion

If we consider this entire series of heterotransplantations of thyroid and cartilage with the surrounding tissues, we come to the conclusion that in all cases the destruction of the transplants has been almost complete at a relatively early date following transplantation. There were up to the tenth day still a few acini exceptionally preserved in the rat-mouse and in the rat-guinea pig transplantations, but not in other, more distant heterotransplantations. Parts of the transplanted cartilage were in some of the hosts still preserved up to twenty days, apparently without regard to relationship. Bone, bone marrow, fat tissue, muscle tissue, and with the few exceptions mentioned, thyroid and parathyroid tissue were always necrotic. In regard to the fat tissue we must make the reservation that when this is densely in-

filtrated with various kinds of cells, it may be very difficult to exclude the possibility that some fat cells may not still be preserved; but in no case could this be definitely established. Even if some cells were preserved, as we found so often in the case of cartilage cells, it is very improbable that these morphological criteria of life implied that the function and metabolism of these cells were normal. We certainly did not observe eight days and later, following transplantation, any growth processes in any of the transplanted tissues, in contrast to what we observe after homoio- and strain transplantation, where regenerative processes in certain tissues are very common. In periods earlier than eight days, we found a few doubtful cases of possible slight proliferative processes in mouse-rat transplants.

It is then this very pronounced necrosis of tissues after heterotransplantation, which evidently makes impossible a definite correspondence between the results of these transplantations, and the phylogenetic relationship between host and transplant. Indications of such a correspondence are perhaps present. Thus we find in the transplantations of cartilage and surrounding tissues from rat to pigeon, on the whole, a more marked infiltration with polymorphonuclear leucocytes than in other kinds of transplantations; but after all the interpretation of such differences is doubtful.

As to the cause of this rapid destruction, there can be no doubt that after heterotransplantation, it is mainly the toxicity of the body fluids which injures tissues directly and which in particular is responsible for the lack of the establishment of a satisfactory connection between the vessels of the host and transplant. These conditions lead to early death of the heterogenous grafts. Contrary to our findings in many cases in homoio- and syngenesiotransplantation, the injurious action of the lymphocytes and connective tissue of the host cannot be held responsible for the death of the transplants. There may be much activity of the connective tissue; but it consists mainly in the invasion of dead tissue and its gradual organization. Also dense collections of lymphocytes are often found; but lymphocytes usually infiltrate the connective tissue, replacing and surrounding the transplanted tissues. They may furthermore infiltrate the interstices of the necrotic fat tissue and even the perichondrium, cartilage, and bone; but all these tissues are by this time either dead or at least very much weakened in their vitality by the injurious conditions of the body fluids of the host; they are not primarily destroyed by lymphocytes. However, as stated, lymphocytes often are present in very large numbers in the fibrous tissue surrounding and replacing the heterogenous grafts.

In our earlier papers on heterotransplantation, we noticed the characteristic appearance of polymorphonuclear leucocytes which contrasted with their usual absence in homoio- and syngenesiotransplantations. In these experiments, we confirmed our previous observations in this respect, but we analysed still further the occurrence of these cells and found it very probable that they are not attracted by the heterotransplant as such, but by the heterogenous tissue when it has undergone necrosis, especially the necrotic fat and thyroid tissue. They usually disappear with the progressive organization of the necrotic tissue, but may still be found in fibrous tissue which has replaced the necrotic material, at a stage when the latter has completely or almost completely disappeared. We may assume that under those conditions they were remnants of the larger number of leucocytes, which had been attracted by the necrotic material at a previous stage. In certain heterotransplants, polymorphonuclear leucocytes may be very significant or altogether lacking; thus they were very inconspicuous after transplantation of cartilage of the cat into the rat. On the other hand, they were often present in very large collections around transplantations of cartilage and adjoining pieces from rat into pigeon. If it is mainly the necrotic material which attracts the polymorphonuclear leucocytes after heterotransplantation, then we must inquire why they are not attracted by the necrotic material which we find after homoiotransplantation, where we usually observed them only in the first few days after transplantation around the grafted tissue, after which period they disappear. We must assume that the autolytic processes, which take place in the necrotic tissue, are not exactly the same in these two types of transplantation; or we may consider the difference in vascularization around and in homoiogenous grafts as the important factor. It seems that the organization of the necrotic material takes place more rapidly after homoiotransplantation than after heterotransplantation. In the past we considered also the possibility that a difference in a possible contamination with microorganisms might be responsible for the presence or absence of these cells. However, the technique used by us in heterotransplantation is the same as that employed in the case of homoiotransplantation, and in the latter, as stated, these accumulations of polymorphonuclear leucocytes are usually absent. Moreover, in special experiments made from this point of view, we did not observe that bacteria, adherent to heterotransplanted epidermis and hair, behaved differently from bacteria adherent to similar material after homoiotransplantation.

We may also conclude that no very distinct differences are found between heterotransplantations which differ in respect to the nearness

or distance of relationship between donor and host, a result that can be readily understood, if we consider the very great intensity of the injury inflicted upon the grafts by heterotransplantations between even nearly related species, such as mouse and rat. However, there are some indications that, also in heterotransplantations, relationship still plays a certain rôle in the results; thus transplantation of pigeon skin to chicken is more favorable than transplantation of pigeon skin to mammals. Furthermore, transplantations of pigeon skin to frogs lead to a rapid destruction of the graft.

In addition, certain differences do exist between the reactions in different species, which function as hosts for the various transplants. Thus it appears that in the mouse and in the guinea pig, the reaction on the part of the polymorphonuclear leucocytes is more intense than in the rat, and there are also indications that, on the contrary, the organizing activity of the connective tissue and the invasion by lymphocytes is stronger in the rat than in the mouse and guinea pig. In the pigeon we found the peculiarity that, in the earlier stages following transplantation, the invasion of necrotic material by polymorphonuclear leucocytes is very marked, while in later stages the accumulation of lymphocytes around the transplant predominates; as in cases of homoiotransplantation in birds, so also after heterotransplantation, follicle-like accumulations of lymphocytes develop in the circumference of the graft. These observations agree also with our former observations, which showed that in the guinea pig and rabbit the leucocytic reaction was more pronounced than in some other species and that therefore reciprocal transplantations may give different results. This was evident, for instance, in the transplantation of guinea pig kidney to pigeon and the reciprocal transplantation of pigeon kidney to guinea pig and also in the transplantation of this tissue to rat.

The outcome is different if we carry out heterotransplantations in the more primitive phylogenetic and ontogenetic stages. Then the reactions against the strange tissues are more moderate, the maximum of response has not yet been reached, when tissues are exchanged between related species, and there is therefore in these cases a more definite correspondence between phylogenetic relationship and the result of the transplantation. Furthermore, in the majority of transplantations in lower organisms we have to deal with a condition analogous to parabiosis, rather than to transplantation in the restricted sense.

Conclusion

1. After heterotransplantation, even between nearly related species, a relatively early necrosis of the grafted tissues occurs, the rapidity

of which varies, however, irrespective of their hosts, in accordance with the resistance of the tissues used.

2. Finer gradations between the results of transplantations in accordance with the phylogenetic relationship of host and transplant cannot be established, the necrosis of the grafted tissues being rapid even in nearly related species serving as hosts; but it is possible that, after heterotransplantations to still farther distant classes, such as amphibians, the mammalian or avian tissues undergo a more rapid and complete destruction than they do after transplantation to other mammalian or avian species.

3. In this destruction, body fluids play the principal part. In general, polymorphonuclear leucocytes appear in larger numbers and for a longer period of time after heterotransplantation, than after homoio- and interracial transplantations; but it is probable that these cells are attracted mainly by certain necrotic heterogenous tissues and they appear with special frequency in certain species. Also lymphocytes gradually appear in larger numbers, particularly in the fibrous tissue surrounding and organizing the transplants.

4. Various species, acting as hosts, differ in the intensity and in the kind of their reactions towards heterotransplants, irrespective of their relationship to the grafts. Reciprocal transplantations may therefore differ as to their results.

ON THE ENERGETICS OF DIFFERENTIATION

II. A Comparison of the Rates of Development of Giant and of Normal Sea-urchin Embryos

ALBERT TYLER[1]

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California)

The following experiments show that normally proportioned giant embryos produced by the fusion of two fertilized eggs develop more rapidly than do normal embryos. This result had been anticipated (Tyler, 1933) on the basis of some experiments on the oxygen consumption and rate of development of half-sized embryos.

Theoretical Part

It has been shown (Tyler, 1933) that the rate of oxygen consumption of two half-sized embryos from isolated blastomeres is the same as that of one normal embryo up to the gastrula stage. The dwarf embryos, however, develop more slowly than the normal. Thus, when compared at the same stage of development (e.g., end of gastrulation), the total oxygen consumption of two dwarf embryos is greater than that of a single normal embryo. The theory upon which these results are interpreted involves the following assumptions: (1) Work is performed (and energy thus required) in the process of gastrulation as in other form changes in embryonic differentiation. (2) The amount of work performed (or energy required) varies directly with the amount of material involved and inversely with some function of the radius of curvature of the embryo or of the part considered. (3) The oxygen consumption is a measure of the energy liberated.

On the basis of assumptions 1 and 2, two dwarf embryos from isolated blastomeres should perform more work (and thus require more energy) for the process of gastrulation than one normal embryo. The experiments show that two dwarf embryos have the same rate of oxygen consumption as one normal embryo. On the basis of assumption 3 this means that energy is liberated at the same rate in the two dwarf embryos as in the normal embryo. The slower development of the dwarf embryo was thus accounted for, since with half the

[1] National Research Council Fellow in the Biological Sciences at the Zoölogical Station in Naples during the early part of this work.

amount of energy available per unit time and with more than half the amount of work to be performed, more time is required to supply the amount of energy necessary for the various processes.

These considerations led to the expectation that giant embryos produced by the fusion of two fertilized eggs should develop faster than the normal embryos.

Experimental Part

Methods of fusing eggs have been described by Morgan (1895), Driesch (1900), Bierens de Haan (1913), Goldfarb (1913, 1915), von Ubisch (1925), Hörstadius (1928) and Balinsky (1932) for sea urchins and by Mangold (1920) and Mangold and Seidel (1927) for salamanders.

Morgan simply removed the membrane shortly after fertilization and found that in a concentrated mass some eggs would fuse in pairs. Driesch treated the eggs with Ca-free sea water after removal of the fertilization membrane and also tried alkaline sea water and centrifuging. Bierens de Haan used essentially the same method. Goldfarb placed eggs in an isotonic or hypotonic solution of NaCl in sea water for considerable lengths of time. Von Ubisch allowed the eggs to remain in Ca-free sea water from the two to four-cell stage, and then brought them close together in ordinary sea water. Hörstadius fused groups of blastomeres with other groups of blastomeres or with whole eggs by bringing them together in a groove in a piece of cinema film in a dish of Ca-free sea water. Balinsky used the same method for fusing two whole eggs in the four-cell stage.

Most of the various methods of fusion were tried. In all the experiments the membranes are first removed either by shaking the eggs in a tube or sucking them up in a pipette one to three minutes after fertilization. If the eggs are fairly concentrated they tend to stick together after removal of the membrane in clumps of various numbers, as noted by Morgan (1895). The eggs are apparently capable of sticking to one another during the period of ten to fifteen minutes after fertilization in which time the ectoplasmic layer forms. If the eggs are packed together by centrifuging after the ectoplasm is formed they do not stick together very readily. Eggs that were fused in pairs either spontaneously or by centrifuging after removal of the membranes were followed in about 300 cases. In most of these, double blastulæ were formed which later separated, giving two normal embryos. In some, double gastrulæ were produced. One normal giant was obtained.

In another way (essentially that of Driesch) the eggs were made

to stick together by placing them for two to five minutes in Ca-free sea water at various times after the formation of the ectoplasmic layer and then packing them together by gently centrifuging immediately after return to ordinary sea water. By this method three normal giants were obtained. The Ca-free sea water treatment and subsequent centrifuging was also applied to eggs in the two-, four- and eight-cell stages. Two normal giants were obtained from eggs so treated in the two-cell stage and 2 more from eggs treated in the four-cell stage out of more than 200 fused eggs that were isolated.

Eggs were handled individually on cinema film according to the method of Hörstadius. Two normal giants were obtained; one from a pair of eggs fused in the two-cell stage and another from a pair fused in the four-cell stage.

The eggs were also handled individually and fused in capillary tubes. A number of capillary tubes sealed at one end are prepared

FIG. 1. Capillary tube for fusion of eggs.

(Fig. 1). The sealed end should have a rounded bottom obtained by blowing as the end is sealed off. About ¾ inch is a convenient length of tube. The tubes are first filled with Ca-free sea water by driving them down in a centrifuge tube containing that solution. They are then mounted vertically in a vial in a Stender dish containing Ca-free sea water so that their open ends are immersed. The eggs are first washed in Ca-free sea water and then, immediately, two eggs are placed in each capillary tube. Into some tubes only a single egg is inserted to serve as a control. The tubes are then transferred to a centrifuge tube containing ordinary sea water and are run at about 2,000 r.p.m. for 15 seconds to 3 minutes. The eggs are thus driven to the bottom of the capillary tube where they may fuse if the tube

is of the proper size and shape. During this time the ordinary sea water gradually diffuses into the capillary tube. If the eggs are allowed to remain in the tube they may develop into swimming blastulæ. It is preferable, however, to remove them at an early stage by

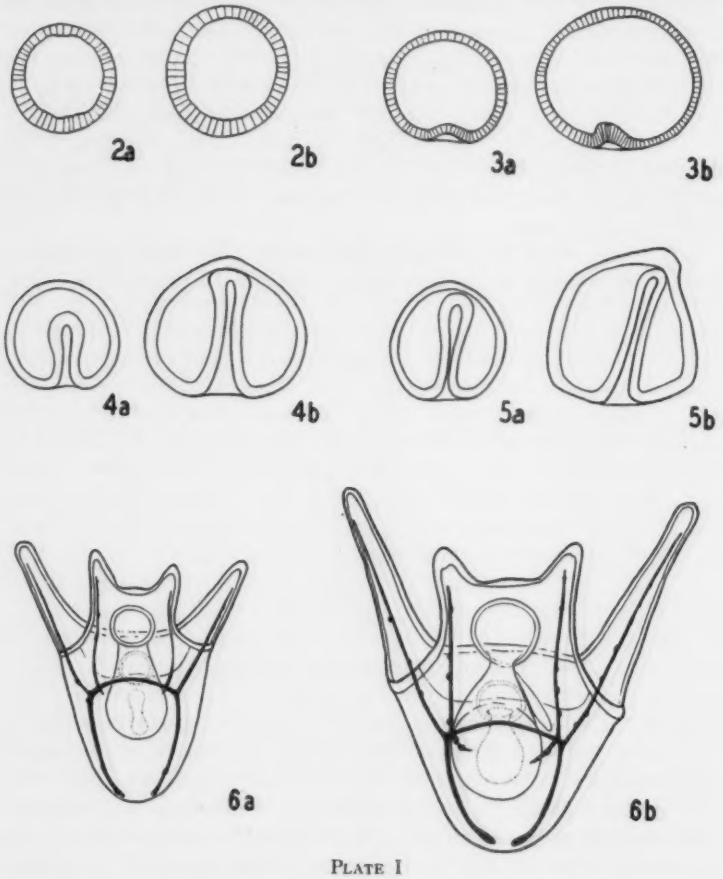

PLATE I

Giants from fused eggs and normal embryos of *Lytechinus*, drawn from photographs of living embryos.

FIGS. 2*a* and *b*. Normal and giant embryos in blastula stage.
FIGS. 3*a* and *b*. Normal and giant embryos in beginning gastrula stage.
FIGS. 4*a* and *b*. Normal embryo about two-thirds gastrulated, giant embryo completely gastrulated.
FIGS. 5*a* and *b*. Normal embryo completely gastrulated, giant embryo in prism stage.
FIGS. 6*a* and *b*. Normal and giant embryo in pluteus stage.

breaking off the sealed end and inverting it in a dish of sea water. The eggs will then slowly drop out. Four normal giants were obtained from more than 150 fused eggs that were followed. Two of them were from eggs fused in the one-celled stage and 2 from eggs fused in the two-cell stage.

A total of 14 normal giants were thus obtained. Of these 10 developed faster than the normal control eggs and 4 developed at the same rate as the controls. The results are presented in Table I. The sea urchins used in these experiments were *Sphærechinus granularis* and *Echinus microtuberculatus* at Naples and *Strongylocentrotus purpuratus* and *Lytechinus* at Corona del Mar, California. The material used and the method of treatment seemed to have no particular relation to the results.

The temperature was not controlled but single eggs treated in the same manner as the fused eggs were isolated in the same dish with the latter. These control eggs are listed with the giant eggs in the table. In four cases (Nos. 4, 7, 8, and 12) the giant and control embryos were found to be developing at the same rate. In the others the giants showed a faster rate of development. The difference in the rate of development is seen at the end of gastrulation and becomes more marked in the later stages. Thus in No. 11 the giant embryo is in the prism stage at a time when the control is still in the gastrula stage; in Nos. 6, 11, and 13 the giants are in the beginning pluteus stage while the corresponding controls are in the prism stage; and in Nos. 1, 3, 6, 11, 13, and 14 the giants are in the pluteus stage while the corresponding controls are entering that stage.

The giant embryos followed in these experiments were those that appeared normal throughout development. Those that showed two invaginations were discarded although they might sometimes give normal plutei according to Bierens de Haan (1913*c*) and Goldfarb (1917). Figures 2 to 6 show some of the giant embryos obtained. In Fig. 4*b* the giant embryo is a finished gastrula while the control embryo shown in Fig. 4*a* is about two-thirds gastrulated. Another giant embryo (Fig. 5*b*) is in the prism stage while the control embryo (Fig. 5*a*) is in the gastrula stage. In Figs. 6*a* and *b* a normal and a giant pluteus at 3½ days are shown. The giant embryo entered the pluteus stage at 64 hours after fertilization and the normal at 82 hours.

The evidence shows then that the giant embryos develop more rapidly than the normal. They complete gastrulation sooner than the controls and the difference in rate of development increases as development continues. The data is not extensive enough to decide whether they both begin gastrulation at the same time, a point that would be of some interest to determine.

TABLE I

The time (in hours) at which various developmental stages of the giant and normal embryos are attained.

	Beginning Gastrula	Completed Gastrula	Prism Stage	Beginning Pluteus	Pluteus
1. *Sphærechinus:*					
Giant	—	22	—	—	50
Control	—	25	—	50	62
2. *Echinus:*					
Giant	—	23	—	41	—
Control	—	25	41	—	—
3. ———					
Giant	16	19	—	50	58
Control	16	21	—	58	65
4. ———					
Giant	—	25	—	—	—
Control	—	25	—	—	—
5. *Strongylocentrotus:*					
Giant	26	30	—	—	—
Control	25	34	—	—	—
6. ———					
Giant	21	26	—	55	66
Control	21	29	55	66	79
7. *Lytechinus:*					
Giant	—	24	—	—	75
Control	—	24	—	—	75
8. ———					
Giant	19	24	—	—	—
Control	19	24	—	—	—
9. ———					
Giant	23	28	—	—	—
Control	25	32	—	—	—
10. ———					
Giant	25	30	—	—	—
Control	25	33	—	—	—
11. ———					
Giant	—	—	34	44	60
Control	—	34	44	60	—
12. ———					
Giant	—	30	41	—	—
Control	—	28	41	—	—
13. ———					
Giant	21	26	35	45	64
Control	22	30	45	64	82
14. ———					
Giant	—	28	39	—	65
Control	—	29	41	65	80

Discussion

The more rapid development of the giant embryos from fused eggs may be interpreted on the same basis as the slower development of the dwarf embryos from isolated blastomeres; namely, that twice

as much energy is available per unit time in the giant embryo, but less than twice the amount of energy is required for gastrulation and other form changes. The giant embryos thus proceed faster in development than the normal embryos.

The slower development of the half-sized embryos has been noted by Morgan (1895, 1903), Driesch (1900, 1908), Hörstadius (1928) and Tyler (1933) for the case of the sea urchin; by Morgan (1901) and Driesch (1903) in *Amphioxus;* by Spemann and Falkenberg (1919) and Fankhauser (1930) in *Triton;* by Tyler (1930) in *Chætopterus.*

To interpret this slower development it has been assumed that a regulation process (whatever that might be) is involved in the formation of a whole embryo from an isolated blastomere and that the regulation requires time. Also to account for the still slower development of the embryos from ¼-blastomeres (Driesch, 1900), it must be assumed that they require even more time for regulation. But when a single embryo is produced from two eggs there certainly must be as much regulation involved as when two embryos are produced from a single egg. On that basis we should expect the giant embryos to develop slower than the normal. Driesch (1900), however, reported that the giant embryo develops as fast as the normal, and the present results show that an even faster rate of development is obtained. Another argument against the assumption that a time interval for regulation accounts for the slower development of the dwarf embryos was presented by Spemann and Falkenberg (1919). They found that in an unequally constricted egg the smaller fragment develops slower than the larger, both being slower than the whole egg. The difference was found to increase during development and was greater than the amount of time that they would care to assume for regulation. They concluded that the factors causing the slower development must be associated with the smaller size of the embryo.

The faster development of the giant embryos from fused eggs has a parallel in the faster development of giant limbs from fused limb-buds reported by Filatow (1932). Filatow grafted a limb-bud of one embryo on to that of another embryo in the same stage. The normal giant limb that developed was considerably further advanced in differentiation than the normal limb of the other side at the times of examination. Although this result in a general way fits in very well with the views expressed here, it is perhaps at present inadvisable to push the parallelism too far.

Another experiment that bears more directly on this work is that of Spemann and Bautzmann (1927). They produced giant and dwarf embryos by cutting two beginning gastrulæ of *Triton* parasagittally,

one to the left, the other to the right of the mid-line, and fused the two larger pieces together as well as the two smaller pieces. In cases in which both fused embryos developed normally, the giant embryo developed markedly faster than the dwarf embryo. It is of interest to note that they say in this connection:—"Das gilt nun ganz allgemein und hängt nicht etwa mit irgendeiner grösseren Schädigung des kleineren Keimes zusammen oder mit einer grösseren Schwierigkeit, die er bei der Regulation zu überwinden gehabt hätte, sondern einfach mit seiner geringeren Grösse und den damit wohl gegebenen grösseren Widerständen bei all den Verlagerungen und Faltungen, mit denen die Entwicklung verbunden ist." This greater resistance in the displacements and foldings of development of which Spemann and Bautzmann speak is one of the main assumptions of the theory developed here (see p. 1) and presented in the earlier work (Tyler, 1933). It is essentially what is meant by the statement (Tyler, 1933, p. 156) that "when similar changes in shape are produced, more work is performed in the case of two half-embryos than in one whole embryo." It is interesting that Spemann and Bautzmann also find this a reasonable assumption to make. It would, however, be well to obtain further justification for this assumption, and a mathematical attempt to do this on purely mechanical considerations is now in progress.

The purpose of studying the energetics of development is to obtain information that would be of value in determining the causes of the form changes. Such information is of value for the problem of differentiation in the same way that a knowledge of the energetics of muscular contraction is of importance in determining the chemical reactions responsible for that process. In the case of differentiation, however, it has been contended that no energy is required for the form changes (Needham, 1932). This conclusion is drawn from the measurements of Meyerhof (1911) and of Shearer (1922), showing a constant ratio of heat production to oxygen consumption throughout development. It was assumed that if energy were required for the form changes then the heat evolved per mole of oxygen consumed should decrease at times when work was being performed. Among the objections that may be raised against this point of view it may be pointed out that heat bears no labels—the energy may have been very useful in producing the form changes before it appeared as heat. There is no more reason for expecting the energy of differentiation to be stored up in the embryo than that it should be stored up in plastic deformation of non-living objects. We conclude from the work on dwarf embryos (Tyler, 1933) and on the giant embryos that energy is required for the form changes. As was pointed out in the

previous paper, the reason for the indirect method of attack is that we may recognize at least two other processes in development, namely maintenance and growth, which may require energy and which it was necessary to eliminate as factors in the result. The problem of obtaining a quantitative estimate of the energy required for differentiation may now be attacked and a number of methods suggest themselves. Among them the effect of temperature on the rate of development and on the rate of oxygen consumption is being investigated since if maintenance, growth, and differentiation should be found to have different temperature coefficients it would help in the analysis of the problem.

A question that has troubled embryologists for some time is why embryos from ⅛-blastomere may develop to the gastrula stage and then stop. As a possible explanation of the failure to develop further, it may be suggested that the tiny embryos do not have enough energy left to go on, but expend it all on the relatively increased work of gastrulation and on the accompanying maintenance and growth requirements.

Summary

1. Giant embryos produced by the fusion of two fertilized eggs develop more rapidly than normal sized embryos.

2. The bearing of this result on the energetics of differentiation is discussed.

3. A new method of fusing eggs is described.

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